

FINAL PERFORMANCE REPORT

1. COVER SHEET

Program: AFOSR, Biomimetics, Biomaterials, and Biointerfacial Sciences

Project title:

Optical, biochemical, and molecular characterization of new bioluminescence systems

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14. ABSTRACT Overall this project has been successful having reached most of the goals originally proposed for the funding period. Screening of light producing organisms (Research Area 1) has been completed in diverse environments, including the Caribbean that was added on a later date. The screening has led to describing the general change with geographical latitude of pigmentation, chemiluminescence and fluorescence in organisms. This has led to documentation of organisms producing light of different color, which could stimulate future research of specific interest for the AFOSR. Also, as a result from this screening, a new Green Fluorescent Protein (GFP) and also been identified from the higher invertebrate (Protochordate) Amphioxus, the most developed invertebrate closest to vertebrate. The discovery of this new GFP (named amphiGFP) raised questions on extent of occurrence of this protein in the Animal Kingdom, considering that GFP had so far been described only from lower invertebrates like jellyfish, corals and some crustaceans.					
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2. OBJECTIVES

This project developed into two areas of research that had complementary main objectives, and each multiple intermediate objectives:

Main objective for Area 1

Screening marine and terrestrial organisms able to produce light by process of bioluminescence, chemiluminescence and/or fluorescence. This area assessed various environments in order to increase diversity of analyzed organisms.

Intermediate objectives:

- 1a: to collect a wide diversity of organisms in various environments
- 1b: to perform visual screening of fluorescence capacity directly from live organisms
- 1c: to trigger bio-/chemi-luminescence of organisms in the laboratory
- 1d: to measure optical properties of bioluminescence and/or fluorescence
- 1e: to provide a listing of species with their light production characteristics
- 1f: to identify species of potential interest for future research

Main objective for Area 2

Identification of molecular structure(s) responsible for light production in organisms already known to be bioluminescent, but with undescribed biochemistry of light production. These organisms are the marine worm *Odontosyllis phosphorea*, the marine brittlestar *Ophiopsila californica*, and an unidentified larval insect from Panama.

Intermediate objectives:

- 2a: to develop field methodology allowing efficient collection of organisms
- 2b: to develop laboratory protocol to enrich and isolate bioluminescent compounds
- 2c: to measure optical properties of the bioluminescence and fluorescence
- 2d: to assess the fundamental biochemistry of the bioluminescence reaction
- 2e: to identify the molecular nature of the bioluminescent compound*
- 2f: to purify and synthesize the bioluminescent compound*

* in collaboration with chemist Bruce Branchini (Connecticut College)

3. SUMMARY

Overall this project has been successful having reached most of the goals originally proposed for the funding period. Screening of light producing organisms (Research Area 1) has been completed in diverse environments, including the Caribbean that was added on a later date. The screening has led to describing the general change with geographical latitude of pigmentation, chemiluminescence and fluorescence in organisms. This has led to documentation of organisms producing light of different color, which could stimulate future research of specific interest for the AFOSR. Also, as a result from this screening, a new Green Fluorescent Protein (GFP) has also been identified from the higher invertebrate (Protochordate) *Amphioxus*, the most developed invertebrate closest to vertebrate. The discovery of this new GFP (named *amphiGFP*) raised questions on extent of occurrence of this protein in the Animal Kingdom, considering that GFP had so far been described only from lower invertebrates like jellyfish, corals and some crustaceans. *AmphiGFP* is most closely related to GFP from crustacean with which it shares about 35% similarity. *AmphiGFP* is currently cloned and expressed for further biochemical, optical, and molecular characterization.

Regarding characterization of light production biochemistry (Research Area 2), significant progress has been made for the marine worm *O. phosphorea*, and marine brittlestar *O. californica*. Both systems involve new reactions of light production, and undescribed compounds able to transfer electronic energy into photons. Both these research topics are currently being continued for a new funding period.

The main data gathered during this project are described here. Some data are currently continued in renewal of this grant; some are complete and available in publications found in the attached appendices section. A total of three scientific publications (accepted or submitted) resulted from this grant period.

This project had a strong public outreach component because of the “magical” nature of light production from organisms. Contact has been established with the popular magazine *Chemical and Engineering News* (<http://pubs.acs.org/cen/>), *National Geographic*, and *Sea and Learn* (Saba, Dutch West Indies) for organizing field trip to view light production from organisms (<http://www.seaandlearn.org/>). A digital underwater movie about fluorescence on coral reefs has been made during research in Panama and is available to the public on www.coralreefmultimedia.org (copy of the movie is enclosed).

4. FINDINGS

This project has generated numerous data, some of which is described in the scientific literature, while some has stimulated further research currently developed as part of renewal of this project.

RESEARCH AREA 1:

Screening for light-producing organisms was carried out in Panama during February 2005, Antarctica during January 2006, Guadeloupe (Caribbean) during December 2006, and Southern California on a regular basis throughout the duration of the project. A total of 78 species of marine invertebrate was analyzed from the tropical environment of Bocas del Toro (Panama), 54

from Guadeloupe (France) in the Caribbean, 57 from the temperate environment of Southern California (La Jolla or Catalina Island), and 34 from the polar environment of Antarctica (analyses completed during polar research training at Mc Murdo station, funded by NSF-OPP).

The screening systematically included information about pigmentation of the tested organism (heavily versus lightly pigmented), its capacity to produce visible fluorescence when exposed alive to blue light (420-440 nm), and its capacity to produce bio- and chemiluminescence (following potassium chloride and hydrogen peroxide exposure, respectively).

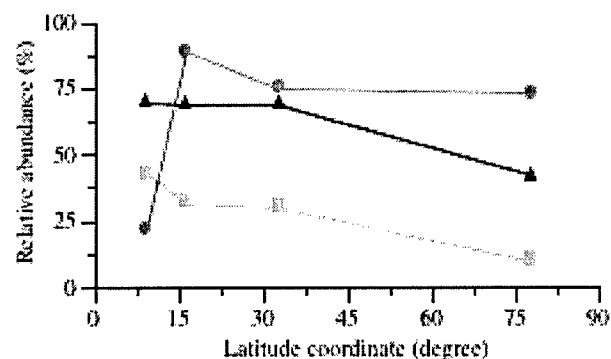
When showing visible fluorescence and when abundant and easy to collect and transport back to the laboratory frozen, the samples were grinded in methanol for spectra analysis of fluorescence (exc. 380 nm). Peaks of spectra were recorded and categorized as related to unidentified compound versus compound related to chlorophyll product. Fluorescence peaks between 380-450 nm were not considered because representing light source or common cell components (e.g., NAD and lipids that produce at 430 and 450 nm).

Most of the tested species have been identified for taxonomic status. The most interesting species, viz. showing intense bioluminescence or fluorescence, have been photographed for documentation of the anatomical distribution of light production.

The research area 1 has reached its goal of providing a listing of light producing characteristics from marine organisms from various environments, while leading to discoveries of new light producing systems.

Data on pigmentation, fluorescence and chemiluminescence are summarized in Figure 1. It indicates that the percentage of species showing visible fluorescence is greater in the tropics than the poles, which follows a trend similar to the one of pigmentation. In contrast, percentage of species with chemiluminescence capacity appears to be always high, except in Panama. This reinforces the hypothesis that fluorescence capacity from pigments could be involved in UV protection while chemiluminescence would be found in other molecular/cellular protective mechanisms, especially against oxidative stress, and thus not related to latitudinal range of UV exposures. The low chemiluminescence in Panama could be due to multiple biological or physical factors other than driven by light exposure. This is in agreement with the literature on fluorescence and pigmentation of corals in the Caribbean, showing different pigmentation in corals between Caribbean islands and Central America (Mazel et al. 2003, *Limnology & Oceanography* 48: 402-411).



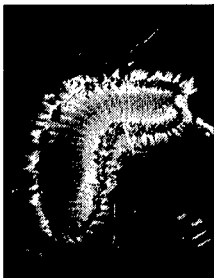

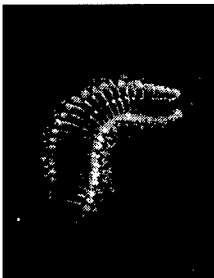

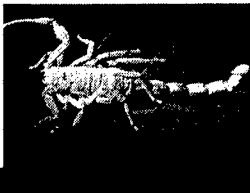
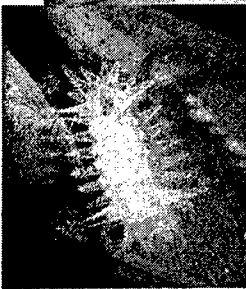
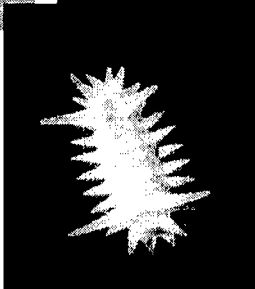

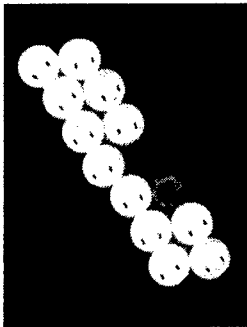
Figure 1: Characteristics of fluorescence (green square), chemiluminescence (red circle), and pigmentation (black triangle) in marine organisms from Panama (9.2°), Guadeloupe (16.2°), San Diego, CA (32.7°), and McMurdo, Antarctica (77.8°).



Intermediate objective 1a: to collect a wide diversity of organisms in various environments

Diversity of marine organisms collected and analyzed was achieved by assessing tropical, temperate and polar environments, but also by collecting samples from various micro-habitats (e.g., soft versus hard bottom), various depths (1 to 30 m), and various ambient light conditions (day versus night). Major phyla were collected, including cnidarians, crustaceans, sponges, mollusks, echinoderms, and worms.

Field screening of bioluminescence and fluorescence was performed visually in the field during night excursions, using blue excitation illumination and an appropriate long-pass filter. In Panama, many species exhibited fluorescence, the most intense ones among marine species being corals, sea anemones, polychaetes and mollusks. In terrestrial species, fluorescence was found in scorpions, caterpillar and insect larvae. This unconventional and pioneering approach has led to an underwater movie and slide show of digital photographs for educational and public outreach purposes. Below is a sample of some of the photographs for marine and terrestrial organisms. For each image pair the left panel shows the organisms under natural light and the right panel shows the organisms producing fluorescence.

Marine organisms	Terrestrial organisms
Ten-ray star coral, <i>Madracis decactis</i> Warty corallimorph, <i>Discosoma sanctithomae</i> Bearded fireworm, <i>Herminobia</i>	Scorpion (species to be identified) Caterpillar larvae (species to be identified) Eggs of insects (species to be identified)
    	     

Intermediate objective 1b: to perform visual screening of fluorescence capacity directly from live organisms

Field screening of fluorescence was performed visually in the field during night excursions (except in Antarctica where the samples were always analyzed in dark room in the laboratory), using blue excitation illumination (420–440 nm) and an appropriate long-pass filter. In Panama, many species exhibited fluorescence, the most intense ones among marine species being corals, sea anemones, polychaetes and mollusks. In terrestrial species, fluorescence was found in scorpions, caterpillar and insect larvae. There were much less samples showing fluorescence in Southern California and Antarctica, and when fluorescence was present, it was usually weak. Data are summarized in Table 1A, 1B, 1C and 1D for Panama, Guadeloupe, Southern California, and Antarctica, respectively (Tables are found in addendum).

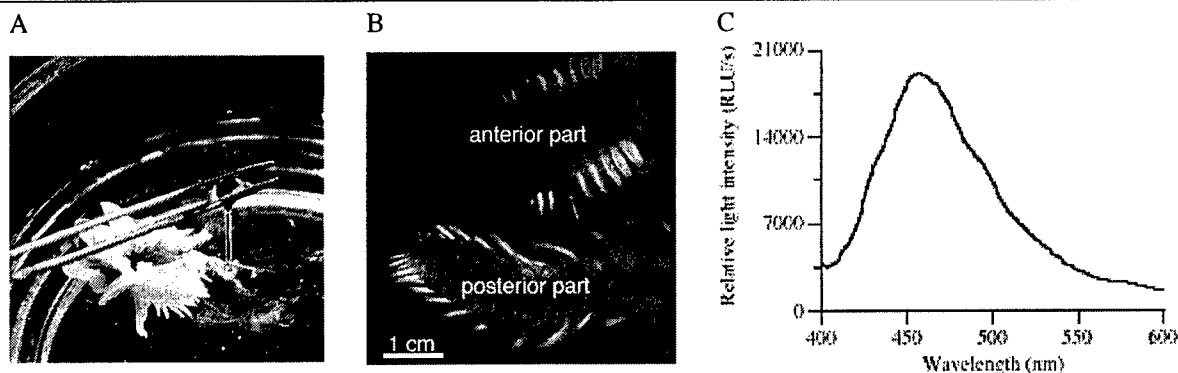
Intermediate objective 1c: to trigger bio-/chemi-luminescence of organisms in the laboratory

Bioluminescence and/or chemiluminescence capacity were tested in the laboratory by triggering light production with potassium chloride and hydrogen peroxide, respectively. Data regarding chemiluminescence capacity are summarized in Table 1A, 1B, 1C and 1D for Panama, Guadeloupe, Southern California, and Antarctica, respectively (Tables are found in addendum).

As for bioluminescence, a luminous fungus has been found to produce a long-lasting glow in the rainforest of Panama. Bioluminescence characteristics of this fungus are described in a manuscript (Publication 1; see addendum).

The marine worm *Chaetopterus variopedatus* has been found in Southern California and described to produce a luminous mucus with light production peaking at 450 nm (Fig. 2). Bioluminescence of this worm has been proposed for further investigation during renewal of this research grant.

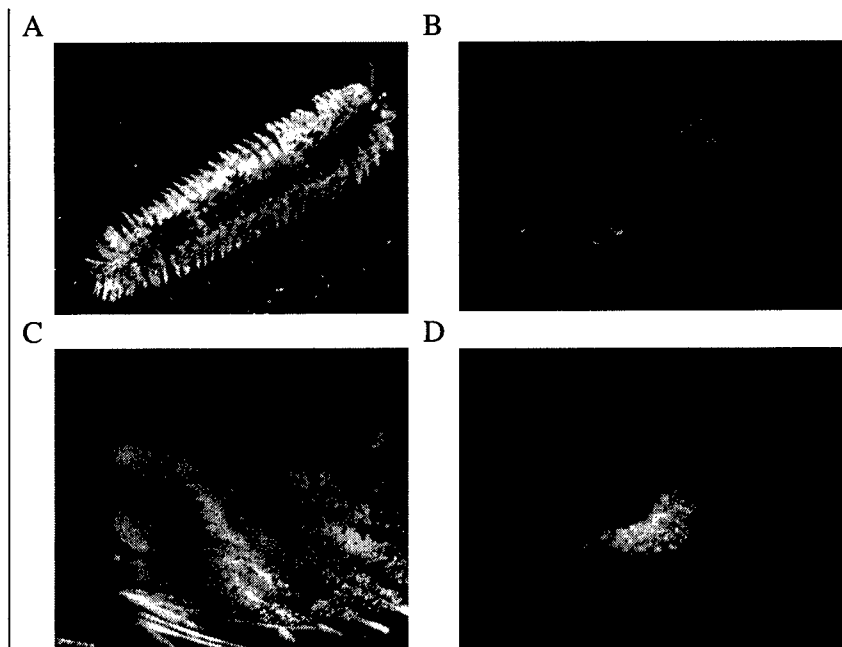
Figure 2: (A) Individual of *C. variopedatus* producing a whitish and sticky mucus upon mechanical stimulation with forceps; (B) multiple areas of anterior and posterior part of the body are fluorescent; (C) light emission from the mucus is a continuous glow, monophasic and peaking at 450 nm.



A marine scale worm (polynoidea, species under identification) from Antarctica has been found to produce intense bioluminescence within each scale (Fig. 3). Only one scale worm

(*Harmothoe lunulata*) from temperate environment has been described in the '80s to produce bioluminescence, and the luminous reagent identified as a unique photoprotein named polynoidin (Nicolas et al., 1982. Photochemistry & Photobiology 35: 201-207). Whether the bioluminescent system of this polar scale worm is similar to the temperate one remains to be assessed.

Figure 3: Unidentified species of a luminous scale worm from Antarctica (worm size is about 2 cm, for scale reference). A: Worm in bright field light; B: worm under epi-fluorescence; C: close-up of scale in bright field light; D: close-up of scale under epi-fluorescence



Intermediate objective 1d: to measure optical properties of the bioluminescence and/or fluorescence

Optical properties were described with the Low Light Digital Spectrograph Echelle SE200. Data regarding peaks of fluorescence spectra are summarized in Table 1A, 1B, 1C and 1D for Panama, Guadeloupe, Southern California, and Antarctica, respectively (Tables are found in addendum). Data show that many species produce fluorescence between 480-550nm, which probably involve GFP-related proteins, and/or novel fluorescent compounds to be identified. Most of the tested species were not bioluminescent, so the fluorescence characteristic most likely is associated with other ecological function of energy transfer, possibly for protection against UV exposure.

Intermediate objective 1e: to provide a listing of species with their light production characteristics

Species with light production characteristics are listed in Table 1A, 1B, 1C and 1D for Panama, Guadeloupe, Southern California, and Antarctica, respectively (Tables are found in addendum). Pictures of the most interesting species (viz. the ones showing intense bioluminescence and/or fluorescence) have also been documented for future research.

Intermediate objective 1f: to identify species of potential interest for future research

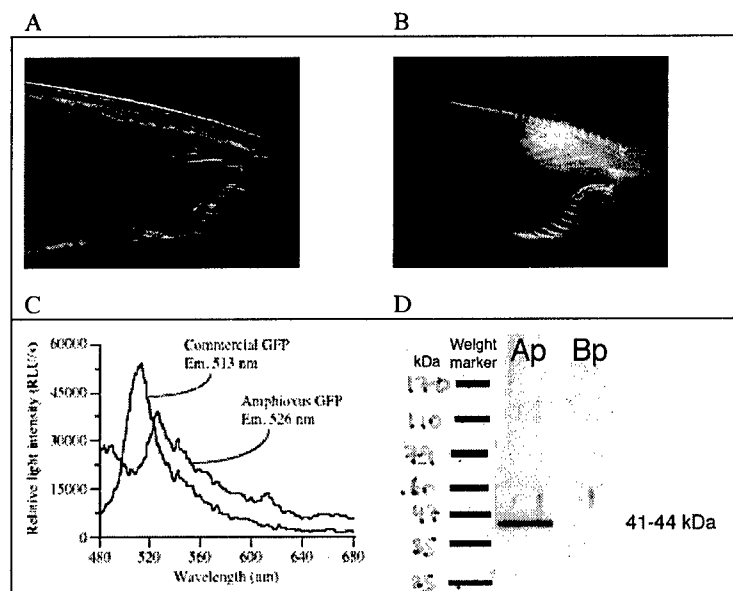
Many species show fluorescence/bioluminescence at wavelengths of potential interest for the AFOSR, ranging from blue to green light. The species that are the most common and showing the most intense light production will be subject to further investigation, as proposed for renewal of this research grant.

A particular interest was given to the fluorescence of the Protochordate *Branchiostoma floridae* (syn. *Amphioxus*). The species is not endemic to Southern California but collected in Florida and maintained in aquarium at SIO by colleagues Drs Nick and Linda Holland. *Amphioxus* specimens show intense fluorescence around the anterior part of the body, around the oral cirri. The fluorescence peaks at 526 nm and is attributed to a Green Fluorescent Protein related to GFP, as determined by GFP-immuno-specific Western Blotting (Fig. 4). This is the first report of the occurrence of GFP in the Protochordate phylum, which opens discussion on the evolution, function and distribution of GFP in Animalia (Publication 2; see addendum).

The GFP of *Amphioxus*, called amphiGFP, has been sequenced as part of this project, following RACE-PCR and cloning. AmphiGFP shows 27 to 72 % similarity with the GFP from Cnidarians, but also about 35 % with the ones from Crustaceans (Table 2; addendum). Preliminary phylogenetic analysis positions the newly discovered amphiGFP as evolutionary related to Cnidarian and Crustacean GFP, probably having common ancestor among lowest invertebrates. The alignment of the GFP *Amphioxus* sequence with the one of *Heteractis magnifica* (Cnidarian) GFP (showing 72 % homology) will allow to identify the conserved sequences through the evolution of GFP in Animalia considering both genomic and proteinic sequences (Table 3).

Phylogenetic analysis shows closer similarity between GFP from *Amphioxus* and Crustacean than with Cnidarian, and comparative analysis of amino acid alignment between amphiGFP (*Amphioxus*) and ppluGFP (Crustacean) shows high degree of conservation for parts of the protein critical for its function in light production (Table 4).

Figure 4: Anterior part of amphioxus in bright field (A) and epifluorescence (B) microscopy. The fluorescence (exc. 420 nm) shows a spectrum shifted to 526 nm (C) compare to commercial GFP at 513 nm. The anterior part contains GFP-like proteins as seen with GFP-immunolabeling on Western Blot (D) using GFP FL-SC-8334 polyclonal antibody against full length GFP of *Aequorea victoria* (Santa Cruz Biotechnology Inc.). Ap: Anterior part; Bp: Main body part.



RESEARCH AREA 2:

Research Area 2 has been productive in terms of generating data and new discoveries, thus achieving its original objective to develop breakthrough research in bioluminescence. The Low-Light Digital Spectrograph purchased during this funding period has been extensively used to characterize light production spectra of organisms (including fluorescence spectra analyzed for Area 1). The Spectrograph has shown optimal performances and has allowed identification of key features in the bioluminescence and/or fluorescence characteristics of luminous organisms. The major progress in this area of research concerns the marine worm *Odontosyllis phosphorea* while the research on the brittlestar *Ophiopsila californica* has focused mainly on establishing an extraction protocol that preserves the activity of bioluminescence/fluorescence.

Marine worm O. phosphorea

Luminous mucus was collected in high concentration to allow isolation of the chromophore (see progress report from colleague Bruce Branchini). Chromatography has been applied to luminous mucus in order to concentrate and purify active reagents involved in the light production. Treatment with inert gases and oxygen scavengers showed that light production is not inhibited by anoxic condition, thus indicating that the light production is associated with a photoprotein system rather than resulting from a typical luciferin-luciferase reaction, as originally described for this organism (Publication 3; see addendum). Biochemical treatments (beta-elimination, PNGase digestion, SDS, DTT, heat denaturing treatments) have assessed the role of the protein versus chromophore component in the light production process. Screening of >80 potential inhibitor/activator substances of the light production process has been completed. It shows unique behavior of the mucus to certain elements, for example to iron that inhibits light production at low concentration. Sequencing and cloning of the most abundant proteins of the luminous mucus have been developed with some promising data, but so far with no definite success.

Brittlestar O. californica

Cations have been screened for their capacity to trigger bioluminescence. Various detergents have been used to extract the luminous activity. Spectrum of fluorescence has been found to be the best marker to trace active component of the light production process during extraction, isolation, and purification. Shift in fluorescence/bioluminescence has been identified, indicating the possible occurrence of a proteinic structure involving energy transfer.

Larval insect from Panama

The organism is small (few mm long) and yet produces a bright and long-lasting spontaneous glow of bioluminescence. The organism seems to be an early stage of firefly larva and has been sent for molecular verification to collaborator Bruce Branchini who has extensive expertise in firefly luciferase.

Intermediate objective 2a: to develop field methodology allowing efficient collection of organisms

Marine worm *O. phosphorea*: An efficient methodology has been developed to collect sufficient numbers of specimens for further study in the laboratory. The worms are now kept alive for several months under aquarium condition, thus allowing extension of the possibility to work on fresh material since the worm only occurs from July to September in the field. The collection process is described in the attached manuscript (Publication 3; see addendum).

Brittlestar *O. californica*: Specimens are collected at night at Catalina Island or Coronado Island and successfully kept alive in captivity for further investigation in the laboratory. Collection occurs by hand during SCUBA diving, quickly scooping brittlestars out of the sand using gardening shovel.

Intermediate objective 2b: to develop laboratory protocol to enrich and isolate bioluminescent compounds

Marine worm *O. phosphorea*: methodology has been established to collect concentrated mucus and is described in a manuscript currently submitted (Publication 3; see addendum). Reagents involved in light production process have also been purified and concentrated through anion-exchange chromatography (DEAE) or size-selective membrane separation (Centricon®).

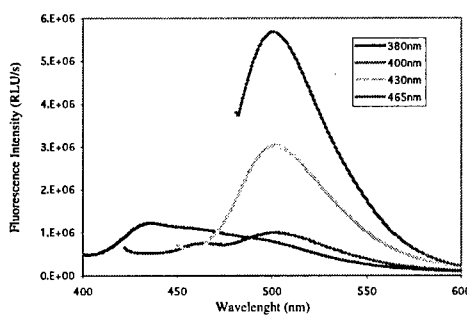
Brittlestar *O. californica*: concentration and partial purification of the luminous reagents was possible through isolation and concentration of light producing cells. Limitation of this approach was that luminous reagents were still in a cellular framework and that mass recovery of cells was not optimal for extensive molecular and chemical work.

An alternative methodology has recently been tested and aimed to extract compounds with fluorescence characteristics similar to the one of bioluminescence. This methodology has been successful and is based on the assumption that the fluorescent compounds are also the ones involved in the bioluminescence process. This methodology will allow tracking of the luminous compounds based on their fluorescence “signature”. It will therefore provide qualitative and quantitative information on occurrence (viz. extraction, concentration, and functionality) of reagents along the various manipulations that will be used to isolate the compounds involved in the bioluminescence reaction (see Intermediate objective 2c).

Intermediate objective 2c: to measure optical properties of the bioluminescence and fluorescence

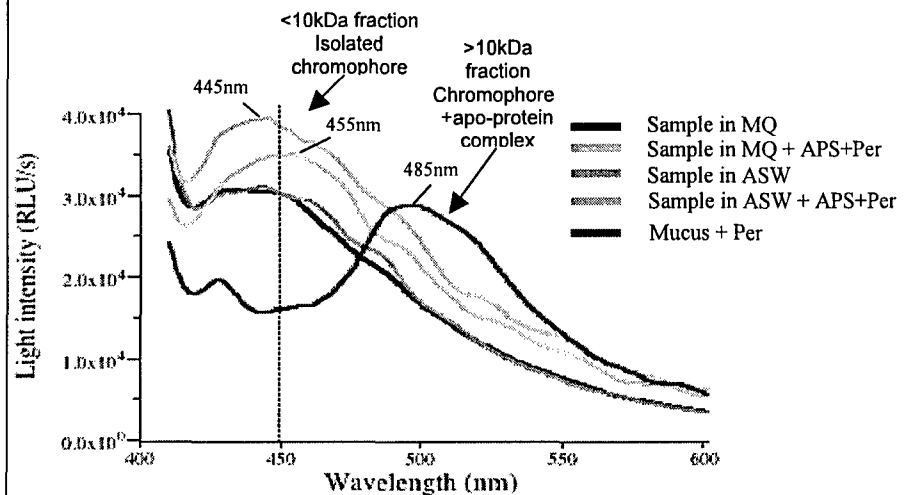
Marine worm *O. phosphorea*: Bioluminescence spectrum is wide and peaking between 494 and 504 nm, which is similar to the fluorescence spectra following excitation at 380 nm when using fresh material (cfr publication in addendum). The fluorescence spectrum, however, is different when using material that has been preserved frozen at -80°C , as identified using a scanning spectrophotometer measuring light production stimulated at various excitation wavelengths (380, 400, 430, and 465 nm) (Fig. 5). Data show that fluorescence of frozen sample is mainly stimulated at longer wavelengths (430 and 465 nm) rather than shorter ones. Meaning of this observation and on the effect of freezing temperature on chemical structure of the mucus remains to be investigated.

Figure 5: Fluorescence spectra of mucus after freezing condition and under various excitation wavelengths (380, 400, 430 and 465 nm). Fluorescence peaks at 494-504 nm as observed for bioluminescence, but only when excited at longer wavelengths (> 430 nm).



Mucus was separated into < and > 10kDa fractions. Fluorescence of these fraction was different, the < 10kDa fraction peaking at about 450 nm while the >10kDa fraction peaked at 485 nm. This was independent of the solvent into which the mucus was dissolved, being MilliQ water or seawater (Fig. 6).

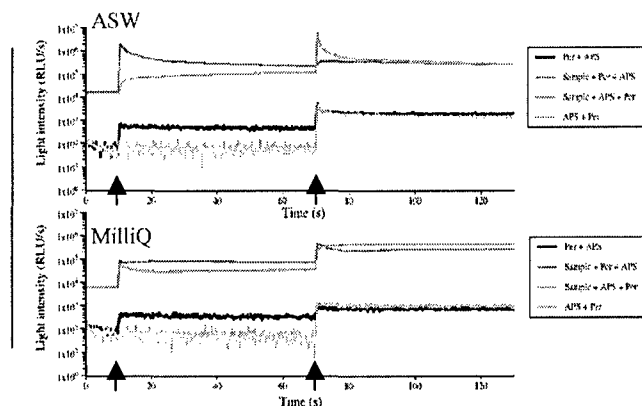
Figure 6: Fluorescence spectra of < 10kDa fraction in different solutions before and after light production (+APS+Per), and from full mucus stimulated to produce light (+Per).



Assuming that the <10kDa fraction represents chromophore isolated from the apoprotein and >10kDa the functional photoprotein system (chromophore integrated into apoprotein), this data suggests the occurrence of energy transfer between the chromophore system and the apoprotein. The apoprotein probably provides adequate moiety for the chromophore to emit light at wavelength that is best transmitted in shallow seawater (480-520 nm), which is crucial for ecological use of bioluminescence by worm individuals.

Fraction >10kDa of luminous mucus has been lyophilized and re-dissolved in artificial seawater or MilliQ water, and the light production stimulated with peroxidase followed by ammonium persulfate, or vice versa. Data show that peroxidase stimulates more light than ammonium persulfate, but also that samples dissolved in artificial sea water produce about 10 times more light than those dissolved in MilliQ water, emphasizing the importance of ions in the surrounding medium of the mucous (Fig. 7).

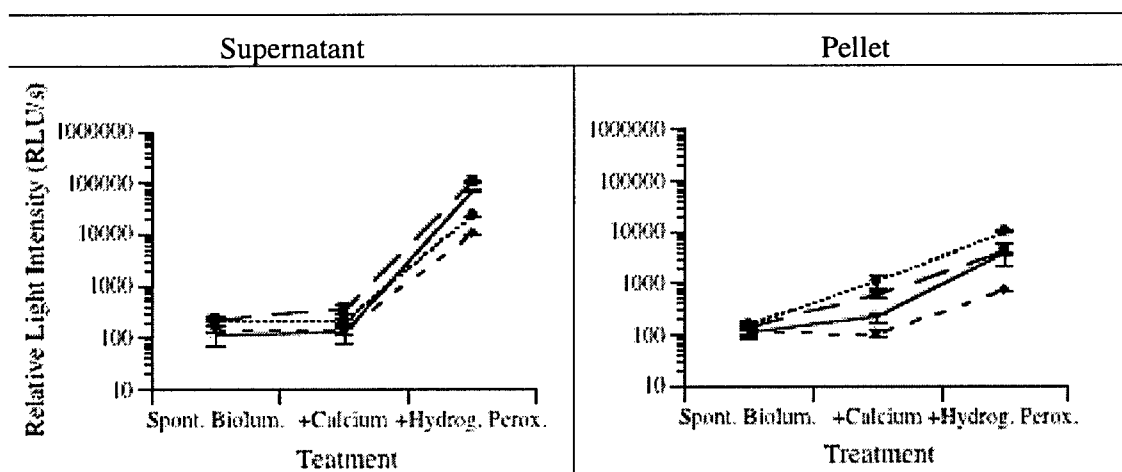
Figure 7: Pattern of light production from lyophilized samples of mucous in artificial seawater (ASW) or MilliQ water (Arrows mark successive times of injection).



Brittlestar *O. californica*: the bioluminescence shows a wide spectrum peaking at 495-515 nm while the fluorescence is shifted, peaking at 518-526 nm (see Figure 9). Because the bioluminescence is soon inactivated when any protocol is used for extraction of the luminous reagents into solution, fluorescence capacity has been chosen as marker of the occurrence of luminous reagents in solution. Different grinding extraction protocols in calcium-free artificial seawater solution (calcium is a co-factor of the light production process and has to be removed to avoid spontaneous bioluminescence) have been tested using various detergents, including SDS, Triton-X and sodium borohydride. Bioluminescence recovery from the supernatant and pellet after grinding extraction has been tested by addition of calcium, thus representative of functional photoprotein, and hydrogen peroxide, representative of functional chromophore (Fig. 8).

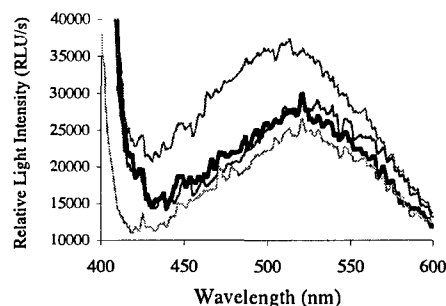
Data indicate that no functional photoprotein was extracted in the supernatant (no light was produced when adding calcium) whereas the chromophore was always extracted, but in greater amount with treatment using calcium-free artificial seawater, and Triton-X. Amount of chromophore left in the pellet was lower than the one present in the supernatant, indicative of good extraction of the chromophore into solution. Calcium-activated bioluminescence in the pellet indicates that some functional photoprotein is still available.

Figure 8: Bioluminescence intensity following addition of calcium and hydrogen peroxide from samples of supernatant and pellet after grinding in calcium-free artificial seawater (———), SDS (.....), Triton-X (— — —), or sodium borohydride (— · — · —)



Fluorescence spectra analysis of the various extractions in supernatant shows a wide spectrum characteristic of the luminous reagent (peaking at ≈ 525 nm), yet with a shift towards lower wavelength (peaking at ≈ 515 nm) for samples treated with sodium borohydride (Fig. 9). This spectrum corresponds to the one of the bioluminescence, suggesting different quality of extraction for sodium borohydride compare to other treatments, which will be further investigated.

Figure 9: Fluorescence spectra (exc. 380 nm) of supernatants following extractions using calcium-free seawater (black line), SDS (blue line), Triton-X (green line) and sodium borohydride (red line). All spectra peak around 525 nm, but sodium borohydride that peaks at 515 nm

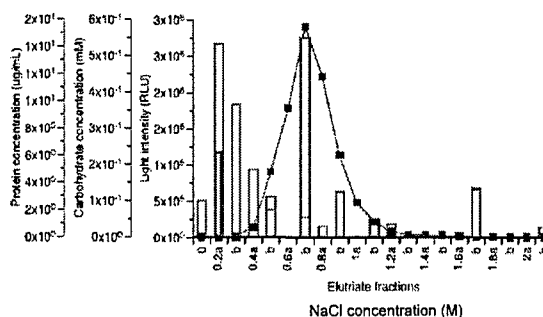


Intermediate objective 2d: to assess the fundamental biochemistry of the bioluminescence reaction

Marine worm *O. phosphorea*: fundamental biochemistry of the bioluminescence reaction was described directly from freshly secreted mucus. Results are described in a manuscript currently submitted (Publication 3, see addendum). In summary, the light production encompasses a wide range of wavelength with a broad peak at 494-504 nm. The bioluminescence process is independent of oxygen and therefore involves a photoprotein and not a luciferin-luciferase system. Intensity of the produced light is increased by exposure to peroxidase or ammonium persulfate, suggesting the involvement of oxydo-reduction processes with sulfate groups as energy source for light production. The photoprotein would involve two entities, one proteinic and the other a chromophore sharing characteristics of possibly a sulfated polysaccharide, or carbohydrate in general (see intermediate objective 2e).

Anion exchange chromatography allowed partial purification of active reagents involved in light production (Fig. 10). Proteinic and carbohydrate measurements of elutriate product showed that fractions with greatest bioluminescence also had greatest concentration in carbohydrate, supporting the direct involvement of a carbohydrate component in the bioluminescence process. Accordingly, elevated levels of galactose, galactose-amine and mannose were found in the mucus.

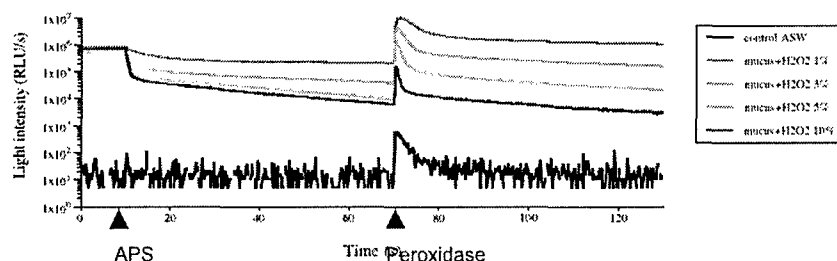
Figure 10: Levels of bioluminescence intensity, protein concentration, and carbohydrate concentration of elutriate fractions following anion exchange chromatography using increasing gradient of sodium chloride for elutriation.



Addition of carbohydrate substrate induced light inhibition, sign of interaction of the exogenous carbohydrate with the light production process through inhibitory competition for a common substrate. A total of 83 substances was tested for effect of possible inhibition/activation on the light production from the mucus (Table 5; cfr addendum). The most interesting results are that iron, copper, silver, cyanide and hydrogen peroxide are strong inhibitors (some of them being known as inhibitors of dehydrogenase/dehydratase activity) while phenylphenol, ammonium persulfate, and peroxidase are strong activators. Calcium does not show any effect on the light production capacity as the case for many photoproteins in marine organisms. Overall, this data indicate that the photoprotein is unique and not sharing any characteristics with known photoproteins.

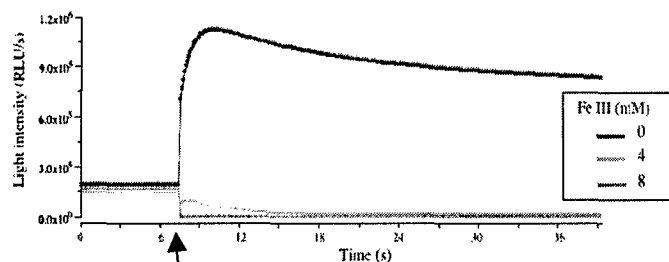
Addition of hydrogen peroxide at concentration as low as 1% affected light production upon exposure to ammonium persulfate as well as peroxidase. The effect was dose-dependent (Fig. 11). This observation goes against the pattern usually observed for light-producing system for which hydrogen peroxide usually triggers light production by oxidation of the chromophore. It indicates however that a co-factor or component of the light production process could be degraded by oxidation and become non-accessible for producing light.

Figure 11: Dose-response inhibition of hydrogen peroxide on luminous mucus. Arrows when ammonium persulfate (APS) and peroxidase were injected.



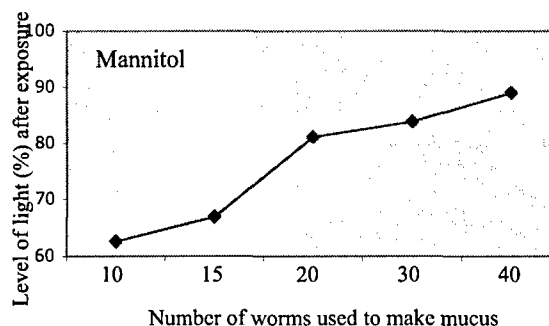
Elements like iron and copper also inhibit spontaneous light production of mucus to concentration as low as few mM (Fig. 12, in the case of FeIII). Iron and copper could be inhibitory co-factors of the light production, which would agree with the fact that the mucus appears blue in color when highly concentrated. In the case of adding seawater to mucus, most of the time it had no effect on light production; sometimes however it would increase light production, suggesting dilution of a component otherwise inhibiting the light production process.

Figure 12: Dose-response inhibition of iron (III) on luminous mucus. Arrow when iron was injected.



Experiments of competitive inhibition were conducted to address whether the substrate of light production involves a carbohydrate related compound. Several carbohydrates (pentoses and hexoses) were used; they were exposed at the same concentration to mucus extracted from different number of worms (10, 15, 20, 30 or 40 worms). Data show that for most carbohydrate there was a dose-dependent (number of worm related) inhibition suggesting competition of light producing system with the added carbohydrate (Fig. 13). Table 5 lists effect of the various products tested on the mucus, by distinguishing four categories: no effect, activator, inhibitor, competitive inhibitor. At this point of the research the mechanisms through which those products affect the light production remains unknown. As for competitive inhibitors like carbohydrate, it still needs to be determined whether the carbohydrate is involved directly in light production as a substrate, or being an intermediary agent involved in light production process, or providing appropriate physico-chemical environment for light production to take place efficiently.

Figure 13: Effect on light production from competitive experiment between mucus and added carbohydrate.



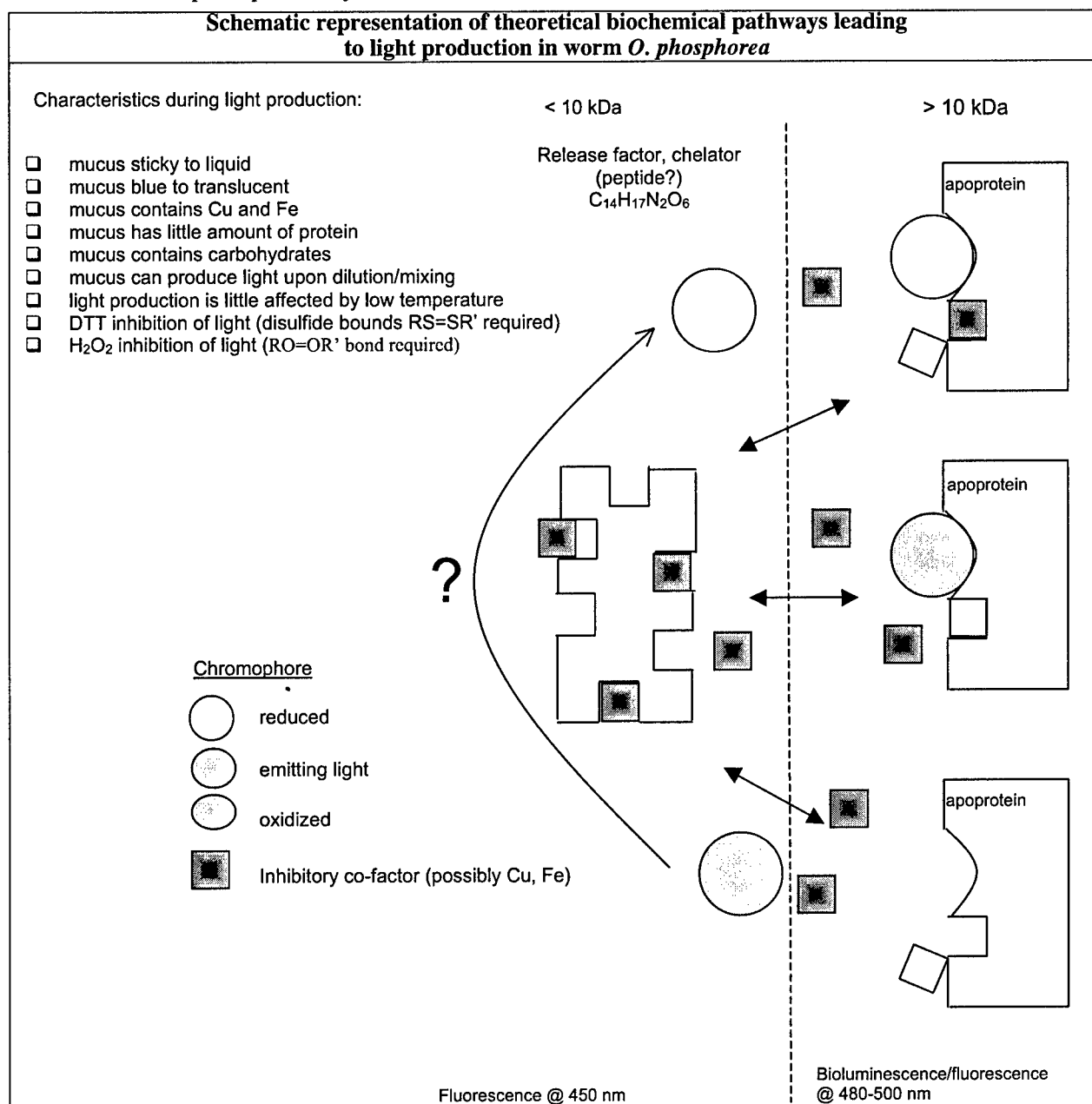
Luminous mucus samples were also treated to identify whether the entity involved in light production was the proteinic and/or the chromophore part of the photoprotein. Four treatments were tested for denaturing the apo-protein: exposure to SDS detergent, dithiothreitol (DTT) to reduce disulfide bounds, beta-elimination process to remove the proteinic entity, and high temperature (100 °C for 30 min). Capacity of fluorescence (exc. 380 nm) and bioluminescence (triggered by peroxidase) were then tested on the treated mucus samples. Data are summarized below. They show that fluorescence capacity is lost after every treatment, suggesting that the apo-protein is involved in absorbing the high-energy source of excitation light, and on transferring the energy to the chromophore for subsequent fluorescence. Bioluminescence however was still possible (yet sometimes to limited extent), reinforcing the statement above, and indicating that the chromophore is a stable inorganic compound.

Potential of fluorescence (exc. 380 nm) and bioluminescence (triggered by peroxidase) after denaturing treatment of the luminous mucus

Treatment	Fluorescence	Bioluminescence
SDS	-	+/-*
DTT	-	+/-*
Beta-elimination	-	+
Heat denaturation	-	+

*the decrease of bioluminescence potential is probably related to denaturation of the peroxidase upon exposure to the sample that still contain the active component of the treatment.

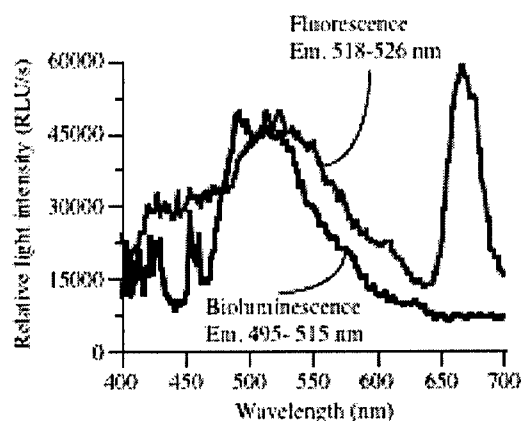
Biochemical pathways leading to light production in *O. phosphorea* are still unknown. Data presented here suggest the occurrence of a release (or inhibitory) factor controlling light production, together with ionic co-factor, which could be iron or copper, both found concentrated in the mucus. The release factor could be the compound found with size <10kDa, being itself fluorescent at 450 nm. The release factor would control access of ionic co-factor to the apoprotein system. This scenario would support the fact that addition of artificial seawater sometimes triggers intense amount of light, probably by dilution of the release factor. It would also allow long-lasting glow with little amount of protein involved and the fact that hydrogen peroxide inhibits light production, which is unusual for photoprotein system.



Brittlestar *O. californica*: The light production process uses calcium as a co-factor that can however be substituted (with various efficiency) by strontium, sodium and cesium. Detergent treatment strongly inhibits light production suggesting that the photoprotein is membranous, or has a trans-membranous domain. Various affinity chromatography will be conducted to further isolate the luminous compound. This will be facilitated by the fact that reagents involved in bioluminescence will now be tracked using their fluorescence capacity that appears preserved through extraction methodology (cfr. Intermediate objective 2c) as opposed to their bioluminescence capacity, which was lost during the first step of extraction into solution.

The prospect of isolating this luminous compound is further exciting because shift in wavelength spectra between bioluminescence and fluorescence (Fig. 14), suggesting the occurrence of a GFP-like protein capable of energy transfer.

Figure 14: Spectrum of bioluminescence following KCl stimulation, and fluorescence (exc. 380 nm) from arm segment of the brittlestar *Ophiopsila californica*. The fluorescence shows a shift of light production at wavelengths greater than the bioluminescence. The fluorescence peak at 670 nm is probably due to the orange pigment characteristic of the brittlestar arm color



Intermediate objective 2e: to identify the molecular nature of the bioluminescent compound

Marine worm *O. phosphorea*: chemical analysis of concentrated mucus samples have lead to the identification of a new fluorescent compound with molecular formulae $C_{14}H_{17}N_2O_6$ and containing carboxymethyl ($-CH_2COOH$) groups (see Dr. Branchini report). This fluorescent compound most likely represents the chromophore since sharing similar spectra of light production.

As for the proteinic part of the photoprotein, SDS PAGE identified two main proteins related to carbohydrate chemistry, namely a glucose dehydrogenase and a mannose dehydratase (data from internal sequencing). Amplification, cloning and sequencing has been developed from RNA of full adult individuals, but with no success because of inhibitory interaction during the PCR process with pigments (adult individuals are indeed deeply pigmented). The methodology has therefore been repeated using juvenile individuals that are not pigmented. RACE-PCR has been successful, yet further specific amplification of the cDNA product for cloning and sequencing will require minute adjustment of PCR conditions. Among possible causes of low specificity is the fact that primers are short (max. 12 bp) and containing multiple sites of possible substitutions. Resolving this issue might require repeating other

internal sequencing analyses from isolated proteins, yet this time from partially purified samples on anion exchange chromatography.

Brittlestar *O. californica*: this objective will be initiated as soon as the luminous compound can be extracted in solution.

Intermediate objective 2f: to purify and synthesize the bioluminescent compound

This objective will be addressed only once cloning of protein(s) involved in light production is completed and *de novo* regeneration of bioluminescence reaction successful when using cloned material and isolated chromophore (expression experiments, currently in progress).

5. PERSONNEL SUPPORTED

Dr. Dimitri Deheyn (PI), Project Scientist, supported at 50%.

Dr. Michael Latz (co-PI), Research Biologist and Senior Lecturer, associated with the research effort, no funding allocated for salary support.

Collaborators

- Dr. Bruce Branchini, chemist, Connecticut College, Connecticut
- Dr. Mark Hildebrand, molecular biologist, UCSD-SIO, CA
- Drs Nick and Linda Holland, molecular biologist, UCSD-SIO, CA
- Dr. Jayantha Gunaratne, carbohydrate chemist, UCSD-SIO, CA
- Dr. Andrew Wier, cell biologist, University of Wisconsin, Madison

Student interns

- Talina Konotchick (UCSD-SIO)
- Magali Porrachia (UCSD-SIO)
- Courtney Cole (UCSB)
- Katherine Wachmann (University of Vienna, Austria)

6. PUBLICATIONS

Deheyn DD, Latz MI (2007). Bioluminescence characteristics of a tropical terrestrial fungus (Basidiomycete). *Luminescence*, in press.

Deheyn DD, Kubokawa K, McCarthy JK, Murakami A, Porrachia M, Rouse GW, Holland ND (2007). Endogenous green fluorescent protein (GFP) from *Amphioxus*. *Biological Bulletin*, in press.

Deheyn DD, Latz MI. Bioluminescence characteristics of the marine polychaete *Odontosyllis phosphorea* (Syllidae). *Invertebrate Biology*. (submitted)

7. INTERACTIONS/TRANSITIONS

a. Conferences/seminars.

Annual Meeting of the American Society for Photobiology, Seattle, USA (July 10-14, 2004). Invited speaker; lecture entitled “Initial characterization of new photoproteins: challenges from field to bench”, which presented some of the results of this project.

International Symposium on Bioluminescence and Chemiluminescence, San Diego, USA (October 15-19, 2006). Lecture entitled “Bioluminescence characteristics of the marine worm *Odontosyllis phosphorea*”.

b. Consultancy. NA

c. Transitions. Invention disclosure (Nov. 29, 2006). amphigFP: a new GFP-like fluorescent protein, UCSD docket # SD2007-120 (Deheyn DD).

8. NEW DISCOVERIES

Results on biochemistry and molecular biology of the luminous system of the marine worm *O. phosphorea* and of the brittlestar *O. californica* are new discoveries.

Green fluorescent protein from the protochordate *Branchiostoma* (*Amphioxus*) is a new discovery because corresponding to the common ancestor form of the GFP from Cnidarians and Crustaceans.

9. HONORS/AWARDS

- Deheyn, DD, NSF-OPP, Polar Research Training Fellowship Award, 2006
- Deheyn, DD, Smithsonian Research Associate Fellow, 2005

Appendix

Deheyn, Final report - FA9550-04-1-0164

Content:

- Table 1A,B,C,D: Listing of light producing invertebrates from various locations
- Table 2: Percent similarity between amphiGFP and other GFPs
- Table 3A,B: Genomic alignment between amphiGFP and Cnidarian GFPs
- Table 4: Protein alignment between amphiGFP (Amphioxus) and ppluGFP (Crustacean)
- Table 5: Listing of inhibitors/activators for light production of worm mucus

- Publication 1: Deheyn DD, Latz MI (2007). Bioluminescence characteristics of a tropical terrestrial fungus (Basidiomycete). *Luminescence*, in press.
- Publication 2: Deheyn DD, Kubokawa K, McCarthy JK, Murakami A, Porrachia M, Rouse GW, Holland ND (2007). Endogenous green fluorescent protein (GFP) from Amphioxus. *Biological Bulletin*, in press.
- Publication 3: Deheyn DD, Latz MI. Bioluminescence characteristics of the marine polychaete *Odontosyllis phosphorea* (Syllidae). *Invertebrate Biology*. (submitted)

Table 1A: Fluorescence and luminescence characteristics of marine organisms collected in Bocas del Toro, Panama.

Group	Species ID	Pigmentation	Visible fluorescence (exc. 420 nm)	Chemiluminescence	different than main body	unidentified compound	Related to chlorophyll/pheophytin pigments
Chordata - Urochordata	<i>Eudistoma sp.</i>	+ (red)	- (?)	-			
Cnidaria - Anthozoa - Hexacorallia	<i>Arachnanthus nocturnus (?)</i>	- (beige)	-	-	All		674
Cnidaria - Anthozoa - Hexacorallia	<i>Cladocera arbuscula</i>	- (beige)	+ (green)	-			
Cnidaria - Anthozoa - Hexacorallia	<i>Bartholomea lucida</i>	- (beige)	+ (red)	-			
Cnidaria - Anthozoa - Hexacorallia	<i>Actinaria sp.</i>	- (brown)	+ (red)	-	Tentacle		619 - 670 - 704
Cnidaria - Anthozoa - Hexacorallia	<i>Actinaria sp.</i>	- (brown)	+ (red)	-	Slime		619 - 670 - 704
Cnidaria - Anthozoa - Hexacorallia	<i>Zoanthus pulchellus</i>	+ (greenish)	+ (green)	-			
Cnidaria - Anthozoa - Hexacorallia	<i>Epilystis crucifer</i>	+ (greenish)	+ (green)	-	Tentacle		609 - 663 - 703 - 734 - 764
Cnidaria - Anthozoa - Hexacorallia	<i>Epilystis crucifer</i>	+ (greenish)	+ (green)	-	Body		674 - 730
Cnidaria - Anthozoa - Hexacorallia	<i>Epilystis crucifer</i>	+ (greenish)	+ (green)	-	Foot		609 - 677 - 730
Cnidaria - Anthozoa - Hexacorallia	<i>Ricordea florida</i>	+ (green)	+ (green)	-	All	479	609 - 664 - 696 - 741
Cnidaria - Anthozoa - Hexacorallia	<i>Stichodactyla helianthus</i>	+ (green)	+ (green)	-	Top	486 - 535 - 572	663 - 726
Cnidaria - Anthozoa - Hexacorallia	<i>Stichodactyla helianthus</i>	+ (green)	+ (green)	-	Bottom		609-696
Cnidaria - Anthozoa - Hexacorallia	<i>Stichodactyla helianthus</i>	+ (green)	+ (green)	-	Tentacle	486 - 527 - 567	625 - 663 - 726
Cnidaria - Anthozoa - Hexacorallia	<i>Discomma sanctithomae</i>	+ (red)	+ (multicolor)	-	All		609 - 664 - 696 - 741
Cnidaria - Anthozoa - Hexacorallia	<i>Viatrix globulifera</i>	+ (red)	+ (red)	-			
Cnidaria - Anthozoa - Octocorallia	<i>Pseudopterogorgia spp.</i>	- (beige)	-	-			
Cnidaria - Anthozoa - Octocorallia	<i>Leptogorgia hebes</i>	- (beige)	+ (+) (red)	-			
Cnidaria - Anthozoa - Octocorallia	<i>Erythropodium caribaeorum</i>	- (beige)	+ (red)	-			
Cnidaria - Anthozoa - Octocorallia	<i>Ellisella barbadensis</i>	- (beige)	+ (beige)	-			
Cnidaria - Anthozoa - Octocorallia	<i>Pseudopterogorgia spp.</i>	+ (beige)	-	-			
Cnidaria - Anthozoa - Octocorallia	<i>Pseudopterogorgia spp.</i>	+ (beige)	+ (red)	-			
Cnidaria - Anthozoa - Octocorallia	<i>Gorgonia flabellum</i>	+ (gray)	-	-			
Cnidaria - Anthozoa - Octocorallia	<i>Gorgonia ventalina</i>	+ (gray)	-	-			
Cnidaria - Anthozoa - Octocorallia	<i>Muriceopsis fluvialis</i>	+ (gray)	+ (green)	-			
Cnidaria - Anthozoa - Octocorallia	<i>Briareum asbestinum</i>	+ (green)	+ (red)	-			
Cnidaria - Anthozoa - Octocorallia	<i>Pterogorgia sp.</i>	+ (orange)	+ (red)	-			
Cnidaria - Anthozoa - Octocorallia	<i>Diadogorgia nodulifera</i>	+ (orange)	+ (red)	-			
Cnidaria - Anthozoa - Octocorallia	<i>Briareum asbestinum</i>	+ (red)	+ (+) (red)	-			
Cnidaria - Hydrozoa	<i>Thysocypus ramosus</i>	- (beige)	- (?)	-			
Echinodermata - Ophiuroidea	<i>Ophiocoma sp.</i>	+ (beige)	-	-			
Echinodermata - Ophiuroidea	<i>Ophiderma paucigranulata</i>	+ (black)	-	-	Arm		
Echinodermata - Ophiuroidea	<i>Ophiocoma echinata</i>	+ (black)	-	-		461 - 486	671
Echinodermata - Ophiuroidea	<i>Ophiderma paucigranulata</i>	+ (black)	-	-	Arm	524 - 542	609 - 667 - 674 - 722
Echinodermata - Ophiuroidea	<i>Ophiocoma wendtii</i>	+ (black)	+ (green)	-	Disk		
Echinodermata - Ophiuroidea	<i>Ophiocoma wendtii</i>	+ (black)	+ (green)	-			
Echinodermata - Ophiuroidea	<i>Ophiothrix suensoni</i>	+ (blue)	-	-			
Echinodermata - Ophiuroidea	<i>Ophiothrix suensoni</i>	+ (brown)	-	-	Arm		539
Echinodermata - Ophiuroidea	<i>(small O. suensoni)</i>	+ (gray)	-	-			
Echinodermata - Ophiuroidea	<i>Ophionereis reticulata</i>	+ (gray)	-	-	Arm		458
Echinodermata - Ophiuroidea	<i>Ophiderma appressum</i>	+ (gray)	-	-	Arm		
Echinodermata - Ophiuroidea	<i>Ophiderma appressum</i>	+ (gray)	-	-			
Echinodermata - Ophiuroidea	<i>Ophiothrix suensoni</i>	+ (green)	-	-	Arm		472
Echinodermata - Ophiuroidea	<i>Ophiothrix suensoni</i>	+ (green)	-	-			
Echinodermata - Ophiuroidea	<i>Ophiactis sp.</i>	+ (orange)	-	-			
Echinodermata - Ophiuroidea	<i>Ophiothrix suensoni</i>	+ (orange)	-	-			
Echinodermata - Ophiuroidea	<i>Bugula minima</i>	+ (beige)	+ (red)	-			
Mollusca - Bivalvia	<i>Lima scabra</i>	+ (red)	-	-			
Mollusca - Gastropoda	<i>Dondice occidentalis</i>	- (gray)	-	-	Slime	539	631
Plantae - Protista - Rhodophyta	<i>Janlia adherens</i>	+ (red)	+ (red)	-			
Porifera - Demospongiae	<i>Dysidea etheria</i>	- (gray)	+ (green/gray)	-	Top	454	670
Porifera - Demospongiae	<i>Dysidea etheria</i>	- (gray)	+ (green/gray)	-	Bottom		674
Porifera - Demospongiae	<i>Ectyoplasia ferox</i>	+ (beige)	-	-	Top	467	616 - 677 - 726
Porifera - Demospongiae	sp. ?	- (black)	-	-			
Porifera - Demospongiae	<i>Niphates erecta</i>	+ (lavender)	-	-			
Porifera - Demospongiae	<i>Aplysina fulva</i>	+ (orange)	-	-			
Porifera - Demospongiae	<i>Plakortis angulospiculatus</i>	+ (orange)	-	-	Foot		670
Porifera - Demospongiae	<i>Plakortis angulospiculatus</i>	+ (orange)	-	-	Top		670
Porifera - Demospongiae	<i>Tethya sp.</i>	- (orange)	-	-			
Porifera - Demospongiae	<i>Pseudoceratina crassa</i>	+ (orange)	-	-			
Porifera - Demospongiae	<i>Diplastrella megastellata</i>	+ (red)	-	-			
Porifera - Demospongiae	sp. ?	-	-	-			
Porifera - Demospongiae	<i>Holopsamma helwigi</i>	-	+ (red)	-	Bottom		674
Porifera - Demospongiae	<i>Holopsamma helwigi</i>	-	+ (red)	-	Top		674 - 730
Porifera - Demospongiae (calc.)	<i>Mycale ditina (calcareous)</i>	+ (yellow)	-	-			
Annelida - Polychaeta - Amphinomida	<i>Hermodice carunculata</i>	+ (orange)	+ (multicolor)	-	Anterior		609 - 664 - 722
Annelida - Polychaeta - Amphinomida	<i>Hermodice carunculata</i>	+ (orange)	+ (multicolor)	-	Posterior		609 - 663 - 711 - 722
Arthropoda - Crustacea	<i>Periclimenes rathbunae</i>	- (beige)	-	-	leg		
Arthropoda - Crustacea	<i>Periclimenes rathbunae</i>	- (beige)	-	-	thorax		
Arthropoda - Crustacea	<i>Synalpheus elisabethae</i>	- (beige)	+ (tip)	-			
Arthropoda - Crustacea	<i>Synalpheus paraneptunius</i>	- (beige)	+ (tip)	-			
Arthropoda - Crustacea	<i>Synalpheus rathbunae</i>	- (beige)	+ (tip)	-			
Chordata - Urochordata	<i>Styela canopus (?)</i>	+ (beige)	+ (orange)	-			
Chordata - Urochordata	<i>Phallusia nigra</i>	+ (black)	-	+	All	472	609 - 670 - 722
Chordata - Urochordata	<i>Phallusia nigra</i>	+ (black)	-	+	Body		
Chordata - Urochordata	<i>Phallusia nigra</i>	+ (black)	-	+			
Cnidaria - Hydrozoa	<i>Sertularia speciosa</i>	- (beige)	+ (red)	+	Body	572	674 - 738
Cnidaria - Hydrozoa	<i>Gymnangium speciosum</i>	- (gray)	-	-			
Cnidaria - Hydrozoa	<i>Halocordyle disticha</i>	+ (gray)	-	-			
Cnidaria - Scyphozoa	<i>Classophora xamachana</i>	+ (green)	+ (red)	+	All		609 - 663 - 703 - 734
Cnidaria - Hydrozoa - Ophiuroidea	<i>Ophiothrix suensoni</i>	+ (orange)	-	+			
Mollusca - Amphineura	<i>Tonicia scharnii</i>	+ (beige)	+ (multicolor)	-	All		609 - 674 - 734
Porifera - Demospongiae	<i>Clathrina canariensis (calc.)</i>	- (white)	+ (red)	+			
Porifera - Demospongiae	<i>Aplysina fulva</i>	- (white/red)	+ (zoanthids)	+	Top		674 - 730
Porifera - Demospongiae	<i>Aplysina fulva</i>	- (white/red)	+ (zoanthids)	+	Middle		674 - 730
Porifera - Demospongiae	<i>Plakortis angulospiculatus</i>	+ (black)	+ (red)	+			
Porifera - Demospongiae	<i>Haliclona sp.</i>	+ (blue)	-	-	All		609 - 674 - 722
Porifera - Demospongiae	<i>Haliclona sp.</i>	+ (blue)	-	-	Bottom		
Porifera - Demospongiae	<i>Holopsamma helwigi</i>	+ (blue)	-	-			
Porifera - Demospongiae	<i>Niphates erecta</i>	+ (gray)	+ (zoanthids)	+			
Porifera - Demospongiae	<i>Iotrochota birutilata</i>	+ (green)	-	+	Top		677 - 722
Porifera - Demospongiae	<i>Iotrochota birutilata</i>	+ (green)	-	+	Bottom		674
Porifera - Demospongiae	<i>Iotrochota birutilata</i>	+ (green)	-	+	Middle	489	674
Porifera - Demospongiae	<i>Agelas clathrodes</i>	+ (orange)	-	+	All		609 - 677 - 722 - 730
Porifera - Demospongiae	<i>Agelas clathrodes</i>	+ (orange)	-	+	Side	460	
Porifera - Demospongiae	<i>Cinchyria sp.</i>	+ (orange)	+ (red)	+			

Table 1A-2: Fluorescence and luminescence characteristics of terrestrial organisms collected in Bocas del Toro, Panama.

Group	Species ID	Pigmentation	Visible fluorescence (exc. 420 nm)	Chemiluminescence	different than main body	unidentified compound	Related to chlorophyll/peophytin pigments
Beetle	Species not determined	+	+	(green/yellow)	All	458 - 533	671
Caterpillar	Species not determined	+	+	(green/yellow)	Posterior	460 - 513	671
Firefly	Species not determined	+	+	(green/yellow)	Posterior	539	
Fly	Species not determined	+	+	(green/yellow)	One	531	
Gnat	Species not determined	+	+	(green/yellow)	One		671
Insect	Species not determined	+	+	(green/yellow)	All	459 - 529	671
Mushroom (Brown)	Species not determined	+	+	(green/yellow)	Piece	533 - 576	
Mushroom (White)	Species not determined	-	+	(green/yellow)	Piece	518	
Scorpion	Species not determined	-	+	(green/yellow)	Tail Section	460 - 500	
Spider	Species not determined	-	+	(green/yellow)	One	516 - 542	625 - 664 - 726
Spider	Species not determined	-	+	(green/yellow)	Leg	539	
Worm	Species not determined	-	+	(green/yellow)	One		664 - 726

Table 18: Fluorescence and luminescence characteristics of marine organisms collected in Guadeloupe, French West Indies, Caribbean.

Group	Species ID	Pigmentation	Visible fluorescence (exc. 420 nm)	Chemiluminescence	Part if different than main body	Fluorescence peaks (exc. 380 nm) detected with Spectrophotograph	
						unidentified compound	Related to chlorophyll/pheophytin pigments
Porifera - Demospongiae	<i>Dysidea</i> sp.	+ (purple)	-	+		515	610-674-695
Porifera - Demospongiae	<i>Helicidona</i> sp.	+ (red)	-	-		524	610-678
Porifera - Demospongiae	<i>Lissodendoryx</i> sp.	+ (green)	-	+		-	618-688-734
Plathelminthes - Turbellaria - Polycladida	<i>Pseudoceros pardalis</i>	+ (orange)	-	+		516	622-678
Cnidaria - Anthozoa - Hexacorallia	<i>Anenome</i> sp. ?	- (brown)	+ (red)	+		-	609-674-725
Annelida - Polychaeta - Sabellida	<i>Sabellastarte magnifica</i>	+ (brown)	-	+		510	609-674-725
Porifera - Demospongiae	<i>Suberites</i> sp.	+ (greenish)	-	+		-	609-677
Porifera - Demospongiae	<i>Spongia</i> sp.	+ (gray)	-	+		513	609-674
Annelida - Polychaeta - Terebellida	<i>Thelepus</i> sp.	+ (red)	- (yellow in periphery)	+		517	671
Chordata - Urochordata - Ascidiacea	<i>Clavelina</i> sp.	-	-	+		510	619-671-711
Cnidaria - Anthozoa - Hexacorallia	sp. ?	-	+ (green)	-	Tentacle	558	609-678-725
Annelida - Polychaeta - Terebellida	<i>Thelepus</i> sp.	+ (red)	-	+		515	615-678-725
Porifera - Demospongiae	<i>Artemisia</i> sp.	+ (brown)	-	+		524	610-695-732
Porifera - Demospongiae	<i>Dysidea</i> sp.	+ (brown)	-	+		486	610-678-725
Porifera - Demospongiae	<i>Iotrochota birotulata</i>	+ (black)	-	+		510	609-678-733
Porifera - Demospongiae	<i>Mycale</i> sp.	+ (red)	-	+		524	609-678
Chordata - Urochordata - Ascidiacea	<i>Ecteinascidia turbinata</i>	+ (brown)	-	+		521	609-678
Porifera - Demospongiae	<i>Tedania ignis</i>	+ (orange)	-	+		-	609-700
Cnidaria - Anthozoa - Hexacorallia	<i>corallimorph</i> sp. ?	-	+ (red)	+		-	619-678-726
Porifera - Demospongiae	sp. ?	+ (black)	-	+		467	609-674
Lophotrochozoa - Sipuncula	<i>Phascolosoma</i> spp.	- (beige)	-	+		524	671-725
Plathelminthes - Turbellaria - Polycladida	<i>Pseudoceros texarus</i>	- (beige)	-	+		517	643
Annelida - Polychaeta - Phyllodocida	sp. ?	+ (orange)	-	+		519	609-671
Echinodermata - Ophiuroidea	<i>Ophiocoma wendtii</i>	+ (black)	-	+		486	671
Echinodermata - Ophiuroidea	<i>Ophiactis</i> sp. ?	+ (gray)	-	+		-	673
Cnidaria - Anthozoa - Hexacorallia	<i>Zoanthus pulchellus</i>	+ (greenish)	+ (yellow)	+	Oral disk	-	-
Cnidaria - Anthozoa - Hexacorallia	sp. ?	-	-	+		-	609-695-726
Mollusca - Bivalvia	<i>Lima scabra</i>	+ (red)	-	+		-	615-678-725
Cnidaria - Anthozoa - Hexacorallia	<i>corallimorph</i> sp. ?	+ (red)	-	+		516-544	622
Cnidaria - Anthozoa - Hexacorallia	sp. ? (sunburst anemone)	- (brown)	+ (yellow)	+	Oral disk	-	610-678-725
Translucent gelatinous spheres (eggs?)	sp. ?	-	-	+		467	610-678-725
Porifera - Demospongiae	<i>Verongula rigida</i>	+ (brown)	+ (yellow)	+	Network	-	-
Lophotrochozoa - Sipuncula	spp. ?	+ (brown)	-	+		467-486	664
Annelida - Polychaeta - Amphinomida	<i>Hermidice carunculata</i>	+ (greenish)	+ (yellow/orange)	+		511	674
Cnidaria - Hydrozoa	<i>Halocordyle disticha</i>	-	-	+		-	609-678-738
Echinodermata - Ophiuroidea	<i>Ophioderma appressum</i>	+ (black)	-	+		520	609-660-725
Arthropoda - Crustacea	<i>Periclimenes rathbunae</i>	-	-	+		-	-
Arthropoda - Crustacea	<i>Brachycarpus biunguiculatus</i>	-	-	+		524	-
Annelida - Polychaeta - Opheliidae	<i>Armandia</i> sp.	-	-	+		516	638
Arthropoda - Crustacea	<i>Neogonodactylus curacaoensis</i>	-	+ (yellow)	+		-	-
Ctenophora - Tentaculata	<i>Mnemioopsis mcgradyi</i>	-	-	+		-	-
Echinodermata - Ophiuroidea	<i>Ophiothrix suensonii</i>	+ (greenish)	-	+		542	622
Echinodermata - Ophiuroidea	<i>Ophiderma rubicundum</i>	+ (red)	-	+		-	609-664-725
Annelida - Polychaeta - Terebellida	<i>Eupolymlia crassicornis</i>	- (beige)	+ (blue)	+	Tentacle	513	-
Porifera - Demospongiae	<i>Pseudoceratina</i> sp.	+ (black)	-	+		522	609-674-725
Cnidaria - Anthozoa - Octocorallia	<i>Erythropodium caribaeorum</i>	+ (purple)	+ (red)	+		-	609-695-725
Cnidaria - Anthozoa - Octocorallia	<i>Pseudopterogorgia</i> spp.	+ (beige)	+ (red)	+		-	610-688-725
Cnidaria - Anthozoa - Octocorallia	<i>Pseudopterogorgia</i> spp.	+ (red)	+ (red)	+		-	618-688-725
Porifera - Demospongiae	<i>Aplysina fulva</i>	+ (orange)	+ (orange)	+	Network	467	670
Porifera - Demospongiae	<i>Ptilocaulis</i> spp.	+ (red)	+ (red)	+		513	610-677-725
Cnidaria - Anthozoa - Octocorallia	<i>Gorgonia flabellum</i>	+ (red)	+ (red)	+		-	688-725
Cnidaria - Anthozoa - Octocorallia	<i>Muricea elongata</i>	+ (red)	+ (red)	+		-	609-688-738
Cnidaria - Hydrozoa	<i>Sertularia speciosa</i>	-	-	-		-	616-688-726
Cnidaria - Anthozoa - Octocorallia	<i>Eunicea</i> spp.	+ (red)	+ (red)	+		-	609-688-725

Table 1C: Fluorescence and luminescence characteristics of organisms collected in San Diego area, Southern California, USA

Group	Species ID	Pigmentation	Visible fluorescence (exc. 420 nm)	Chemiluminescence	Part if different than main body	Fluorescence peaks (exc. 380 nm) detected with Spectrograph	
						unidentified compound	Related to chlorophyll/pheophytin pigments
Chordata	Branchiostoma floridae	-	- (head)	-			
Arthropoda - Thoracica	Tetractilia rubescens	- (light brown)	+	-			
Chordata - Ascidiacea - Corellidae	Corella willmeriana	-	-	-	All	-	
Chordata - Urochordata	Metandrocarpa taylori	+	+	-			
Chordata - Urochordata	Didemnum carinatum	+	+	-			
Cnidaria - Anthozoa - Hexacorallia	Pachyactinia fimbriatus	+	+	-	All		616 - 664 - 719
Cnidaria - Anthozoa - Hexacorallia	Zoanthid anemone	+	+	-			
Cnidaria - Anthozoa - Hexacorallia	Anthopleura sola	+	+	-	tentacle	497 - 534 - 568	
Cnidaria - Anthozoa - Hexacorallia	Balanophyllia elegans	+	+	-			
Cnidaria - Anthozoa - Octocorallia - Gorgonacea	Leptogorgia chilensis	+	+	-			
Cnidaria - Hydrozoa - Hydroids	Tubularia crocea	+	+	-	Polyp	551	616 - 664 - 719
Echinodermata - Crinoidea	Florametra serratissima	+	+	-	Piece		616 - 664 - 719
Echinodermata - Ophiuroidea	Ophioreis annulata	+	+	-			
Echinodermata - Ophiuroidea	Ophiotrix spiculata	+	+	-			
Echinodermata - Ophiuroidea	Gorgonocephalus eucnemis	+	+	-	Arm		616 - 664 - 719
Echinodermata - Ophiuroidea	Ophiotrix sp?	+	+	-	Arm		616 - 664 - 719
Echinodermata - Spinulosida - Echinasteridae	Henricia leviuscula	+	+	-			
Porifera - Demospongiae	Ophiletes pinnata	+	+	-			
Annelida - Polychaeta	Ancistrosyllis pinnata	+	+	-			
Annelida - Polychaeta	Spirorbis sinuatus	+	+	-	mouth fluo		
Annelida - Polychaeta	Scale Worm	+	+	-	All	493 - 548	616 - 664 - 719
Arthropoda - Malacostraca	Alpheus clamator (Snapping shrimp)	+	+	-			
Arthropoda - Malacostraca	Alpheus clamator	+	+	-			
Arthropoda - Thoracica	Tetractilia rubescens	- (light brown)	+	-			
Bryozoa - Gymnolemata	Hippodiplosia insculpta	- (light brown)	+	-			
Bryozoa - Gymnolemata	Zoobotryon verticillatum	- (light brown)	+	-		516 - 542	616 - 671
Bryozoa - Gymnolemata	Bugula neritina	+	+	-			
Chordata - Urochordata	Metandrocarpa taylori	+	+	-			
Cnidaria - Anthozoa - Hexacorallia	Corynactis californica	+	+	-			
Cnidaria - Anthozoa - Hexacorallia	Balanophyllia elegans	+	+	-			
Cnidaria - Anthozoa - Octocorallia - Gorgonacea	Muricea fruticosa	+	+	-		513	616 - 671 - 726
Cnidaria - Hydrozoa - Hydroids	Plumularia sp.	- (white/green)	+	-		460 - 539	
Cnidaria - Hydrozoa - Hydroids	Tubularia crocea	+	+	-	Stalk		616 - 664 - 719
Echinodermata - Holothuroidea	Parastichopus parvimensis	+	+	-	epidermis		
Echinodermata - Holothuroidea	Parastichopus parvimensis	+	+	-	Head	491 - 513	
Echinodermata - Ophiuroidea	Amphipolis squamata	-	+	-	Arm	518 - 539	616 - 671 - 726
Echinodermata - Ophiuroidea	Amphipolis squamata	-	+	-	Disk		616 - 664 - 719
Echinodermata - Ophiuroidea	Ophioreis annulata	+	+	-	Arm	505 - 513	616 - 664 - 719
Echinodermata - Ophiuroidea	Amphipolis pugetana	+	+	-	Arm	518	671
Echinodermata - Ophiuroidea	Amphipolis pugetana	+	+	-	Disk	516	609 - 671 - 719
Echinodermata - Ophiuroidea	Ophiotrix spiculata	+	+	-			
Echinodermata - Ophiuroidea	Ophiopellis californica	+	+	-	Arm	471 - 539	664
Echinodermata - Ophiuroidea	Ophiopellis californica	+	+	-	Arm	518 - 539	
Echinodermata - Spinulosida - Echinasteridae	Henricia leviuscula	+	+	-			
Echinodermata - Valvatida	Linckia columbiana	+	+	-			
Mollusca - Gastropoda	Antipopeia barbarensis	+	+	-	mouth fluo		
Mollusca - Gastropoda - Limpet	Megathura crenulata	+	+	-	Piece of mantle	572	616 - 664 - 719
Mollusca - Gastropoda - Limpet	Collisella scabra	+	+	-			
Mollusca - Gastropoda - Nudibranchia	Spurilla liviae	+	+	-			
Plathelminthes - Turbellaria - Polyclad	Prostheceraeus bellistriatus	+	+	-			
Porifera - Demospongiae	Ophiletes pinnata	+	+	-			
Porifera - Demospongiae	Aplysia flatularis	+	+	-			
Cnidaria - Anthozoa - Hexacorallia	Epizoanthus scotinus	+	+	-	All		616 - 664 - 719
Cnidaria - Anthozoa - Hexacorallia	Teslia crassicornis	+	+	-	All		
Cnidaria - Anthozoa - Hexacorallia	Diadumene leucolea	+	+	-	Piece	526	616 - 664 - 719
Cnidaria - Anthozoa - Hexacorallia	Metridium sp	+	+	-	Arm	495	616 - 664 - 719
Echinodermata - Ophiuroidea	Ophioplocus esmarki	+	+	-	Piece of mantle		616 - 664 - 719
Mollusca - Gastropoda	Norritia norriti	+	+	-	All	471 - 521 - 560	616 - 664 - 719
Mollusca - Gastropoda - Nudibranchia	Cadlina florumaculata	+	+	-	Piece		616 - 664 - 719
Porifera - Calcarea	Leucetta sp	+	+	-	All	521	616 - 664 - 719
Porifera - Demospongiae	Toxodonta sp.	+	+	-	All		

Table 1D: Fluorescence and luminescence characteristics of marine organisms collected in McMurdo, Antarctica.

Group	Species ID	Pigmentation	Visible fluorescence (exc. 420 nm)	Chemiluminescence	Part if different than main body	Fluorescence peaks (exc. 380 nm) detected with Spectrograph	
						unidentified compound	Related to chlorophyll/pheophytin pigments
Cnidaria - Hydrozoa	sp.?	-	-	-		539 - 570	671
Echinodermata - Crinoidea	<i>Promachocrinus kerguelensis</i>	+ (brown)	+ (orange)	-			616 - 677 - 749
Echinodermata - Ophiuroidea	<i>Ophiurolepis brevisima</i>	-	-	-	Arm		671
Echinodermata - Ophiuroidea	<i>Ophiurolepis sp.</i>	-	-	-	Arm		664 - 749
Mollusca - Gastropoda	<i>Marseniopsis mollis</i>	+ (yellow)	-	-		486 - 516 - 539	671
Mollusca - Pteropoda (sea butterfly)	<i>Clione antarctica</i>	+ (beige)	+ (head)	-		486 - 544	
Nudibranch	<i>Tritonia antarctica</i>	-	+ (head)	-	Posterior	500	671
Porifera	<i>Leucetta leptoraphis</i>	-	-	-	Arm	516	664
Amphipod	<i>Abyssorchomene plebs</i>	+ (beige)	+ (mouth)	+		516	664
Annelida - Polychaeta	<i>Flabelligera mundata</i>	- (light beige)	- (chaetes only)	+	Posterior	513	616 - 726
Annelida - Polychaeta	sp? (<i>Aglaophamus trissophyllus</i>)	+ (brown)	+ (cirri, yellow)	+	Posterior		616 - 671 - 726
Annelida - Polychaeta	Polynoid polychaete sp?	+ (brown)	+ (yellow/green)	+	Scale		
Arthropoda - Chelicerata	<i>Colossendeis australis</i> (Giant seaspider)	+	-	+	Leg	518	616 - 671
Arthropoda - Chelicerata	<i>Pentanymphe antarcticum</i>	+	+ (green)	+	Leg		616 - 677 - 738
Arthropoda - Crustacea	Arcturid isopod	-	+ (head, yellow/green)	+	Posterior	556	616 - 671
Arthropoda - Crustacea	<i>Wellnerium wellneri</i> (stalked barnacle)	-	-	+	Body		616 - 671
Arthropoda - Crustacea	<i>Metopoides sp.</i>	-	-	+	Body	499 - 539	
Arthropoda - Crustacea	<i>Glyptonotus antarcticus</i> (Isopod)	+ (beige)	-	+	Leg	516	664
Arthropoda - Crustacea	<i>Bathylasma corolliforme</i> (sp.?) (Barnacle)	-	-	+	Posterior		616 - 674 - 738
Chordata - Ascidian	<i>Cnemidocarpa verrucosa</i>	+	-	+			616 - 671 - 726
Cnidaria - Hydrozoa	<i>Ophiodes arboreus</i>	-	-	+			616 - 723
Cnidaria - Hydrozoa	<i>Clavularia frankliniana</i>	-	-	+			616 - 664 - 738
Cnidaria - Hydrozoa	<i>Urtichopsis antarctica</i> (sp.?)	-	-	+		516	616 - 671
Cnidaria - Hydrozoa	unidentified sp.	-	-	+		539	664
Cnidaria - Hydrozoa	unidentified sp. 2	-	-	+			671
Echinodermata - Asteroidea	<i>Notasterias armata</i>	- (light orange)	-	+		527	671
Echinodermata - Asteroidea	<i>Odontaster validus</i>	+ (orange)	-	+	Arm	459 - 518	671
Echinodermata - Ophiuroidea	<i>Ophiurolepis sp.</i>	-	-	+	Disk	458	616 - 671 - 726
Ectoprocta (Bryozoan)	<i>Reteporella sp.</i>	-	-	+		572	616 - 671 - 726
Ectoprocta (Bryozoan)	<i>Camptopiles sp.</i>	- (brown)	+ (yellow)	+		486	616 - 677 - 738
Nemertean	<i>Parborlasia corrugatus</i>	+ (brown)	+ (mouth)	+		549	622
Porifera	<i>Sphaerostylus antarcticus</i>	-	-	+		516	616 - 677 - 726
Porifera	unidentified sp.	+ (orange)	-	+			616 - 671 - 726 - 749
Porifera	<i>Dendrilla antarctica</i> (Sp. ?)	+ (yellow)	+ (orange)	+		472 - 516 - 556	

* Data missing because the organism, also newly found to be bioluminescent, is currently used for taxonomic identification

Table 2: Similarity in percent (%) between *Amphioxus* GFP and GFP sequences available in the literature. Most of the sequences are from Cnidarians while some are from Crustaceans. E values (expectations values) represent the number of different alignments with scores equivalent to that expected to occur in a database search by chance. The lower the e value, the more significant the score.

Species	GenBank ID	e values	Sequence length	Similarity (%)
CNIDARIA				
<i>Aequoria victoria</i> 1	M 62653	-		-
<i>Aequoria victoria</i> 2	M 62654	-		-
<i>Discosoma striata</i>	AF 168420	e^{-60}	209	43
<i>Zoanthus</i>	AF 168422	e^{-036}	197	31
<i>Aequoria macrodactyla</i> 1	AY 013824	e^{-008}	124	31
<i>Anemonia sulcata</i> 2	AF 322221	e^{-045}	201	35
<i>Dendronephthya</i> sp. 1	AF 420591	e^{-064}	204	36
<i>Ricorda florida</i> 1	AY 037772	e^{-048}	207	34
<i>Aequoria macrodactyla</i> 2	AF 435427	e^{-008}	124	33
<i>Agaricia fragilis</i> 1	AY 037765	e^{-049}	205	36
<i>Renilla reniformis</i> 1	AF 372525	e^{-029}	193	29
<i>Ptilosarcus</i> sp. 1	AY 015995	e^{-034}	192	31
<i>Anemonia sulcata</i> 4	AF 545827	e^{-045}	201	35
<i>Heteractis magnifica</i> 1	AY 157666	e^{-045}	190	72
<i>Ricordea florida</i> 2	AY 037773	e^{-047}	207	41
<i>Discosoma</i> sp. 2	AF 272711	e^{-061}	210	37
<i>Montastraea cavernosa</i> 7	AY 181552	e^{-059}	206	34
<i>Zoanthus</i> sp. 2	AF 168423	e^{-035}	197	32
<i>Aequoria victoria</i> 3	L 29345	-		-
<i>Aequoria victoria</i> 4	X 83959	-		-
<i>Aequoria victoria</i> 5	X 83960	-		-
<i>Aequoria victoria</i> 6	E 17099	-		-
<i>Anemonia majano</i> 1	AF 168421	e^{-043}	208	36
<i>Anemonia sulcata</i> 1	AF 246709	e^{-050}	199	35
<i>Heteractis crispa</i> 1	AF 363776	e^{-047}	207	55
<i>Condylactis gigantea</i> 3	AY 037777	e^{-044}	206	50
<i>Cerianthus</i> sp. 1	AY 296063	e^{-026}	197	35
Phialidium sp.	AY 485333	e^{-008}	134	-
Hydrozoa (unidentified)	AY 485334	e^{-014}	219	30
Hydrozoa (unidentified)	AY 485335	e^{-012}	208	31
Hydrozoa (unidentified)	AY 485336	e^{-016}	193	27
CRUSTACEA				
<i>Pontellina plumata</i>	AY 268071	e^{-006}	54	47
<i>Pontellina plumata</i>	AY 268072	e^{-006}	54	49
<i>Labidocera aestiva</i>	AY 268073	e^{-006}	163	46
<i>Pontella meadi</i>	AY 268074	e^{-006}	164	44
<i>Pontella meadi</i>	AY 268075	e^{-006}	164	48
Copepoda (unidentified)	AY 268076	e^{-008}	164	47

Table 3A: Alignment between the *Amphioxus* EST genomic sequence bbne101k07 and the GFP genomic sequence from *Heteractis magnifica* (Cnidaria)

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AmphEST_bbne101 ga-----acaagctgtcttcccgagttcatccgaga-----ccagcaaagatcc
Heteractis      gattttggacagctgttcaaccaggcaaattcaagaagtcacatctttatctctcagtc
                **          *****      *   *   *   *   *   *   *   *
AmphEST_bbne101 gcgcaaccatgtctctcc-----ctacgagtcacgaccttcacatcttcggctcc
Heteractis      aggaaaatgtattcttacatcaaagaaccatgcgcagtaagggtttacatggaaggaaat
                * * * * *   *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 gtcaatggcgcggagttcgacctggtggggaggcggaagggaacccgaacgatggaacg
Heteractis      gttaacaaccacgcctttaagtgcactgcagaaggagaaggaaaaccatacaaaggctca
                ** * *   *   *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 ctgga---gaccagtgtgaaatccaccggggcgccctgcc--ctgctcccgtgctga
Heteractis      caaaaactgacgattaccgtaactgaaggaggtcctctgccatttgctttcgacattctt
                *   *   *   *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 tcggacccaacctgggttacggcttctaccagtacctgcccttccctggcg--cctc
Heteractis      tcgcacgc--ctttcagtagtgcaataagggtgttcaccaagttaccccgacgatattctg
                *** * * *   *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 acccttccaaaccgccatcacggacggagggtaccaggttcaccgtgtgttcaagtttga
Heteractis      atttctttaagcagtctctttctggagggttttacttgg--aaaagagtaagcaactatga
                *   *   *   *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 agacggcggtgctgaattgcaacttccgctacacctacgagggcgatcaaa-----gg
Heteractis      ggacggaggagtccctaccgttgacaaaaaactagtctggaggagattgcattatttg
                ***** * * *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 ggagttccagctgatcgggtcaggtttccctgccggcgccctgtgatgtccggcggaact
Heteractis      caacattaaagtacatggcactaacttccccgcagatggtccggtgatgc---aaaaaca
                *   *   *   *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 gaccaccctggacaggagc-----gtggccaaactgca
Heteractis      gacca--atggatgggagccatcaactgaaactgttattccacggggtgaaggaattctg
                ***** * * *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 gtgctcgg-acgaccgcaccatcacgc-gcactaac-----
Heteractis      ctgcgcgatgtgcccgactgaagctgcgtaataacaaaggacatctctctgtgcatg
                *** * *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 -----aactggagcttctgcaccac
Heteractis      gaaacaacttacaagccaaacaaaagggtgaacctgccaaaactgcattttcatcatttg
                ***** * * *   *
AmphEST_bbne101 cgatgggaagcg-----acaccaggcggacgtt
Heteractis      cgaatggagaaggatagtagtgacgatgagaagaccattaagcagcacgaggatgtg
                *** * *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 cagacgaactacaccttcgc-----caagccgctcccgccg
Heteractis      agggcaagctacttcaatgtgcgctttgatgagagctcgtaaatgatcatttccttattg
                * * * * *   *   *   *   *   *   *   *   *   *
AmphEST_bbne101 gtctcaaggagaagatg-----ccgatcttctggggcac-----
Heteractis      atttcaatgttagggcattcagtttccaaattttcttatacacagtcatttcccttcgta
                * * * * *   *   *   *   *   *   *   *
AmphEST_bbne101 -----
Heteractis      gcctacttaccatgttttgttgaagtcaataaacagctaagcccaaaaaaaaaaaaaa

```

CLUSTAL (-like) formatted alignment by MAFFT (v5.860)

Table 3B: Alignment with the frame 2 for *Amphioxus* EST protein sequence and the protein sequence of *Heteractis magnifica* (Cnidaria)

	10	20	30	40	50	60
Heteractis	MYSYIKETMRSKVYMEGNVNNHAFKCTAEGEGKPYKGSQKLTITVTEGGPLPFAFDILSH					
AmphEST	----MSLPTSHDLHIFGSVNGAEFDLVGGGKGNPNDDGTLETSVKSTRG-ALPCSPLLIGP					
Prim.cons.	MYSY2222222222G2VN222F22222G2G2P22G2222222T2GG2LP2222222					
	70	80	90	100	110	120
Heteractis	AFQYGNKVFTKYPDDIPDFFKQSLSGGFTWKRVSNYEDGGVLTVDQKTSLEGDCIICNIK					
AmphEST	NLGYGFYQYLPFPGGASPFQTAITDGGYQVHRVFKFEDGGVLNCFRITYEGD--QRGV					
Prim.cons.	222YG2222222P22222F222222GG2222RV222EDGGVL2222222EGDCI22222					
	130	140	150	160	170	180
Heteractis	VHGTNFPADGPVMQKQTNGWEPSTETVIPRGEIGILLRDVPALKLRNNKGHLLCVMETTYK					
AmphEST	ADRVRFPCRRACDVRRTD--HPGQE----RGQTAVLG-----					
Prim.cons.	22222FP22222222T2GW2P22ETVIPRG2222L2DVPALKLRNNKGHLLCVMETTYK					
	190	200	210	220		
Heteractis	PNKRVNLPKLHFHHLRMEKDSISDDEKTIKQHEDEVASYFNVRFDESS					
AmphEST	---RP-----HHHRH-----					
Prim.cons.	PNKR2NLPKLHFHH2R2EKDSISDDEKTIKQHEDEVASYFNVRFDESS					

Alignment data :
Alignment length : 228
Identity (*) : 41 is 17.98 %
Strongly similar (:) : 29 is 12.72 %
Weakly similar (.) : 28 is 12.28 %
Different : 130 is 57.02 %
Sequence 0001 : Heteractis (228 residues).
Sequence 0002 : AmphEST (151 residues).

Comparison between protein GFP sequences identified and the Florida *Amphioxus* EST database

Query_id	Subject_id	% identity	e-value
gi 28627998 gb AA016871.1	bbne101k07	72.73	1.2e-06
gi 28627998 gb AA016871.1	bblv034m06	32.65	1.3e-06
gi 28627998 gb AA016871.1	bbne112g21	27.19	1.3e-06
gi 28627998 gb AA016871.1	bbne153i21	27.59	1.7e-06

Table 4: Amino acid alignments of GFP between amphiGFP (amphioxus, Protochordate) and ppluGFP (*Pontellina plumata*, Crustacean).

amphiGFP ppluGFP	<p>MSLP¹TAHDL¹HI¹FGSVNGAEFDLVGGGKGNPNDGTLET¹SVKSTRGA</p> <p>-MPAMKIECRITGT¹LN¹GVEBELVGGGEGTPEOGRMTNKNKSTKGA</p> <p>LPCS¹PL¹LIGPNLGYGFYQYL¹PPPGG-ASPFQTAITDGGYQVHRVF</p> <p>LTFS¹PYLLSHVMGYGFYHFG¹TYPSGYENPELHA¹INNGCYTNTRIE</p> <p>KEEDGGVLN¹CNFRYTYEGGKIKGEFQLIGSGFPAGGPVMSGGLTT</p> <p>KYEDGGVLHVSESYRYEAGRVIGDFKVVGTGFPEDSVIETDKAIR</p> <p>LDRSVAKLQCSDDCTITGTNNWSECTTDGKRHQADVQIN¹YIEAKP</p> <p>SNATVEHLHPMGDNVLVGSFART¹SLRDGGYYSFVVD¹SHMHEKSA</p> <p>LP-AGLKEKMPIFLGHQIEVKASKTEITLCEKVKAFIDTV---</p> <p>IHP¹SILQNGGPMFAFR¹RVEELHSNTELGI¹VEYQHAKTPIAFA</p>
---------------------	---



Identical amino acid



Conserved substitution



B-sheets of barrel structure



Caps of barrel structure

Table 5: Remaining level of bioluminescence (%) from the luminous mucus after addition of a potential inhibitor/activator substance. Substances were always added at final concentration of 10 mM, or 15 % concentration when using acids/bases.

Substance	Chemical formula	Level of bioluminescence (%)	Type of interaction*
Sulfuric acid	H ₂ SO ₄	0	Inhibitor
Ferric chloride	FeCl ₃	0	Inhibitor
Salicylic acid	C ₇ H ₆ O ₃	0	Inhibitor
Sodium bisulfate	NaHSO ₄	0	Inhibitor
Aminonaphtol	C ₁₀ H ₉ NO ₇ S ₂	0	Inhibitor
Methylhydrazine	CH ₆ N ₂	0	Inhibitor
Hydrogen peroxide	H ₂ O ₂	0	Inhibitor
Sodium cyanide	NaCN	2	Inhibitor
Succinic acid	C ₄ H ₆ O ₄	2	Inhibitor
2,4 Dichloro-6-nitrophenol	C ₆ H ₃ Cl ₂ NO ₃	2	Inhibitor
Mercury chloride	HgCl ₂	4	Inhibitor
Silver Chloride	AgCl	5	Inhibitor
Copper Chloride	CuCl ₂	7	Inhibitor
EDTA 50 mM	C ₁₀ H ₁₆ N ₂ O ₈	13	Inhibitor
EDTA 10 mM	C ₁₀ H ₁₆ N ₂ O ₈	20	Inhibitor
Nitrophenol	C ₆ H ₅ NO ₃	30	Inhibitor
Hydroxylamine	NH ₂ OH	35	Inhibitor
Orcinol	C ₇ H ₈ O ₂	42	Inhibitor
Sodium thiocyanate	NaSCN	48	Inhibitor
Colchicine	C ₂₂ H ₂₅ NO ₆	50	Competitive inhibitor
Sodium ferric cyanide	Sodium Fe CN	52	No clear trend effect
AICAR	C ₉ H ₁₅ N ₄ O ₈ P	56	Competitive inhibitor
4-Hydroxyindole		56	Inhibitor
Mercaptoethanol	C ₂ H ₆ OS	57	Inhibitor
Pyruvate	C ₃ H ₃ O ₃ ⁻	57	No clear trend effect
ATP	C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	57	Inhibitor
NADH	C ₂₁ H ₂₉ N ₇ O ₁₄ P ₂	59	Competitive inhibitor
MethylPhenylPropanol	C ₁₀ H ₁₄ O	61	Competitive inhibitor
Benzamidine	C ₇ H ₈ N ₂	62	Competitive inhibitor
Sodium ThioSulfate	H ₁₀ Na ₂ O ₈ S ₂	64	Competitive inhibitor
Streptomycin	C ₂₁ H ₃₉ N ₇ O ₁₂	66	Competitive inhibitor
Benzoquinone fresh	C ₆ H ₄ O ₂	67	Activator
Glucose dehydrogenase		67	Inhibitor
Heparin	C ₁₂ H ₁₉ NO ₂ OS ₃	67	No clear trend effect
5-Hydroxyindole		67	Inhibitor
Sodium carbonate	Na ₂ CO ₃	68	No clear trend effect
Fucose	C ₆ H ₁₂ O ₅	71	No clear trend effect
NAD+	C ₂₁ H ₂₇ N ₇ O ₁₄ P ₂	71	Competitive inhibitor
Lactose dehydrogenase		71	Competitive inhibitor
Phenol	C ₆ H ₆ O	71	No clear trend effect

Table 5 - continued

Galactose	$C_6H_{12}O_6$	72	Competitive inhibitor
Swainsonine	$C_8H_{15}NO_3$	72	No clear trend effect
Lactate	$C_3H_6O_3$	73	No clear trend effect
Sorbitol	$C_6H_{14}O_6$	74	Competitive inhibitor
Guanine	$C_5H_5N_5O$	74	Inhibitor
Deoxymannojirimycin	$C_6H_{13}NO_4$ HCl	74	No clear trend effect
Starch	$C_{12}H_{22}O_{11}$	74	Competitive inhibitor
DMSO	C_2H_6OS	74	Competitive inhibitor
Arsenic chloride	AsCl	76	Competitive inhibitor
Phenylmercury acetate	$C_8H_9HgO_2$	76	Competitive inhibitor
Sodium sulfate	Na_2SO_4	77	Competitive inhibitor
Ammonium sulfate	$N_2H_8SO_4$	78	Competitive inhibitor
Lithium chloride	LiCl	79	Competitive inhibitor
Acetazolamine	$C_4H_6N_4O_3S_2$	80	Competitive inhibitor
Ammonium Chloride	NH_4Cl	80	Competitive inhibitor
Arabinose	$C_5H_{10}O_5$	81	Competitive inhibitor
Sodium Iodine	INa	81	No clear trend effect
Mannitol	$C_6H_{14}O_6$	81	Competitive inhibitor
Benzoquinone old	$C_6H_4O_2$	83	No clear trend effect
Sodium Chlorate	$ClNaO_3$	84	Competitive inhibitor
PhenylMethane sulfate		85	No clear trend effect
Glucose	$C_6H_{12}O_6$	86	Competitive inhibitor
Mannose	$C_{24}H_{42}O_{21}$	87	Competitive inhibitor
Imidazole	$C_3H_4N_2$	87	No clear trend effect
Nitrobenzylamine	$C_7H_8N_2O_2$	88	Competitive inhibitor
Sodium pyrophosphate	$Na_4O_7P_2$	91	No clear trend effect
Adenosine di-phosphate	ADP	92	No clear trend effect
Gum xanthan		97	No clear trend effect
MilliQ		100	No clear trend effect
Xylose	$C_5H_{10}O_5$	101	Competitive inhibitor
PAPS		101	No clear trend effect
Nitrophenyl phosphate	$C_{18}H_{32}N_3O_6P$	110	No clear trend effect
Piperine	$C_{17}H_{19}NO_3$	110	No clear trend effect
Nifedipine	$C_{17}H_{18}N_2O_6$	112	No clear trend effect
ASW		113	No clear trend effect
Sulfanilamide	$C_6H_8N_2O_2S$	125	Activator
Anthrone	$C_{14}H_{10}O$	133	Activator
Phenylphenol	$C_{12}H_{10}O$	202	Activator
Sodium persulfate	$Na_2O_8S_2$	217	Activator
Ammonium persulfate	$N_2H_8(SO_4)_2$	236	Activator
Alcian blue	$C_{56}H_{68}Cl_4CuN_{16}S_4$	293	Activator
Peroxidase		454	Activator

* *Inhibitor* , when effect was systematically $\leq 75\%$ of original light emission; *No clear trend effect* , when effect was variable with number of worms; *Competitive inhibitor* , when effect was relative with number of worms; *Activator* , when light emission was systemetically $\geq 125\%$



**Bioluminescence characteristics of a tropical Terrestrial fungus
(Basidiomycetes)**

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**Bioluminescence characteristics of a tropical
Terrestrial fungus (Basidiomycetes)**

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ABSTRACT

Freshly collected samples of luminous mycelium of a terrestrial fungus from Panama were investigated for their bioluminescence characteristics. Taxonomic identification of fungal species could not be determined because of the lack of fruit bodies. Fluorescence excited by 380 nm illumination had an emission spectrum with a main peak at 480 nm and additional chlorophyll peaks related to the wood substrate. Bioluminescence appeared as a continuous glow that did not show any diel variation. The light production was sensitive to temperature and decreased with temperatures higher or lower than ambient. Bioluminescence intensity was sensitive to hydration, increasing by a factor of 400 immediately after exposure to water and increasing by a factor of one million after several hours. This increase may have occurred through dilution of superoxide dismutase, which is a suppressive factor of bioluminescence in fungus tissue. The mycelium typically transports nutritive substances back to the fruit body. The function of luminescent mycelium may be to increase the intensity of light from the fungus and more effectively attract nocturnal insects and other animals that serve as disseminating vectors for fungal spores.

KEYWORDS: Terrestrial fungus; Firefly; Glow wood; Bioluminescence;
Ecological function

INTRODUCTION

Bioluminescence, the production of visible light by living organisms, involves different intensities, colors and patterns depending on the species, and is associated with an ecological function of intra- and/or inter-specific visual communication for attracting mates or prey, or for defense against predation (1, 2).

In the terrestrial environment, bioluminescence in fungus is visually fascinating with extensive anecdotal reports. Luminous fungi belong to the Basidiomycetes and are found in tropical as well as temperate environments, always associated with hydrated substrates. Therefore, luminous fungi are commonly found growing on decaying wood or leaves, leading to the popular name of “foxfire” or “glow wood” when their bioluminescence is visible at night. Bioluminescence is produced by the mycelium, the fruit-body part, or both, depending on the species (3, 4). Glow wood, however, refers to the expansive mycelium of the fungus that covers the degrading substrate and not the fruit-body part of the fungus.

Luminous fungi are among the few terrestrial organisms to produce long-lasting spontaneous bioluminescence, the others including insects and bacteria. In insects, only the larval stages of some flies and beetles, commonly referred to as glowworms, exhibit spontaneous glowing (5-7). Terrestrial luminous bacteria belong to one species, *Photorhabdus* (*Xenorhabdus*) *luminescens*, which is found in soil but grows mainly on organic substrates such as the body of dead organisms, or infests the gut of insects for which they represent a pathogenic factor (8, 9).

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6 Despite their wide popularity, luminous fungi have been far less subject to
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8 scientific investigation than other luminescent organisms. Luminous fungus have
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10 been grown in the laboratory for scientific and/or commercial purposes, and several
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12 studies have focused on identifying culture conditions optimal for growth and
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14 bioluminescence display. High water content of the compost powder is essential for
15
16 optimal growth, optimal growth occurs at 27 °C and pH 4 for several species and is
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18 accompanied with the most intense bioluminescence, and bioluminescence remains
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20 steady for at least 30 days under optimal conditions (10-13). Bioluminescence
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22 intensity decreases after exposure to certain contaminants and fungal light production
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24 is therefore used in the laboratory to test toxicity of soils (14). The chemistry of the
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26 light reaction is different from all other known bioluminescent systems and has not
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28 yet been fully characterized (3, 15, 16). It is a typical enzyme-substrate (luciferin-
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30 luciferase) reaction that involves superoxide anion (O_2^-) and a sesquiterpene
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32 substrate, and appears to be controlled by superoxide dismutase (17, 18).
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39 Based on anecdotal field observations of fungus bioluminescence reported on
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41 the world-wide-web, light production occurs as a steady glow that has different
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43 intensities depending on location, wood substrate, and time of year. There is little
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45 known about the light emission from fungus collected directly from the field (19).
46
47 This study characterizes the bioluminescence intensity of fungus as a function of
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49 time of day, temperature, and extent of hydration. The results are discussed in the
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51 context of the possible ecological function of bioluminescence in fungus.
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MATERIALS AND METHODS

Mycelia of luminous fungus were hand collected between January 20 and February 7, 2005 at night in the rainforest of Bocas del Toro, Panama. Fungi were obtained from decaying pieces of wood showing bright bioluminescence. The light was yellow and long lasting and seemingly unaltered during the process of collection. The pieces of wood were then placed in clean Zip-Lock® bags and brought back to the Smithsonian Tropical Research Institute (STRI) for analysis. There, the areas of bright bioluminescence were isolated from the rest of the wood using a scalpel. Usually, this required removing the top 1 to 3 mm layer of wood, below which the bioluminescence appeared much dimmer.

Emission spectrum of fluorescence. Chips of decaying wood with intense bioluminescence and of living wood with no bioluminescence were stored at -80°C and transported to Scripps Institution of Oceanography (USA). There, samples of wood were scraped for removal of outer and deeper layers and the material immersed in 99 % methanol for chemical extraction. After removal of any solid material by centrifugation, the fluorescence spectrum of the methanol extract was measured using a Low Light Coupled Intensified SE200 Spectrograph (Catalina Scientific Instruments, Tucson, AZ). The excitation wavelength was 380 nm and the fluorescence emission spectrum was measured during 10 s duration exposures. Spectra were smoothed using the built-in smooth function, which applies a triangle-

function convolution filter to the spectrum, with settings of 1,000,000 counts, 5 pixels, 20 nm, and 40 cm⁻¹.

Day/night variation of bioluminescence. Three chips of wood (α , β , and χ) of similar size ($L \times W \times H \approx 7 \times 4 \times 1$ mm) were collected from different parts of the wood with distinct light intensity at the STRI laboratory. They were weighed ($\alpha > \beta > \chi$, initial weight at 0100 local time), placed in individual pre-weighed glass tubes, and measured for light emission ($\alpha > \beta > \chi$, initial bioluminescence at 0100 local time) using a Sirius luminometer (Berthold Detection Systems), which exhibits a dark level varying between 0 and 20 RLU/s. Weight of the tubes containing the wood piece and bioluminescence were followed for up to 73 h keeping the tubes at room temperature and under natural variations of ambient light. At each time point the weight of the wood chip was calculated by the difference with the weight of the empty tube initially measured. This approach avoided touching the wood directly, which could alter light emission. At each time point the bioluminescence intensity was recorded for 2 min and expressed as mean values of relative light units (RLU/s).

Effect of hydration on bioluminescence. To assess short-term effects of hydration on light production, a set of experiments consisted of measuring bioluminescence of chips of wood at room temperature continuously for 30 min, where 50 μ l of MilliQ water was added at time 10 s. To assess long-term effects, at the end of the 73 h experiment assessing the day/night variation in bioluminescence,

a drop of water was added to the same α , β , χ chips of wood and bioluminescence recorded after 9 h.

Effect of temperature on bioluminescence. Small chips of luminous wood ca. 2 mm in size were mixed together to form a stockpile of luminous wood “granules”. Using a micro-balance (Sartorius, R160P), sub-samples of 0.1 g of the stockpile were weighed and placed into transparent polyethylene tubes to which a few drops of water were added. This allowed homogeneity in size and shape among the samples, as well as their hydration. The samples were then maintained at -20 , 4 , 15 , 25 , 37 , or 60 °C and bioluminescence measured for 2 min at time 0, 0.15, 0.30, 1, 2, and 6 h. In between time points, the tube was sealed to limit dehydration.

Collection of fungus and analyses of weight and bioluminescence were performed several times during the two-week period of investigation yet only one representative dataset is presented here. Replicates showed identical results except for the initial level of light intensity.

RESULTS

The fluorescence emission spectrum showed multiple peaks (Fig. 1). Peaks at 615, 680, and 730 nm are known to correspond to chlorophyll *a*, chlorophyll *b*, and phaeophytin, respectively. Samples of glowing wood also had a peak at 480 nm, which is believed to be associated with the bioluminescence emission. In samples of outer wood showing bright bioluminescence, the 480 nm was equal in intensity to the

chl *a* peak and had a full width half maximum of 120 nm. Samples of outer wood that did not display bioluminescence lacked the 480 nm peak although the chlorophyll peaks were still present. Samples of deeper wood showing dimmer bioluminescence had a lower intensity to the 480 nm peak.

The three samples of wood had distinct weight being 0.082 g for sample α , 0.053 g for β , and 0.048 g for χ . Bioluminescence was more homogenous being 2.1×10^7 RLU/s for sample α , 2.1×10^7 RLU/s for β and 2.0×10^7 RLU/s for χ . Bioluminescence was a steady glow that slowly decreased to 90 % of the original intensity of light production after 10 h, and 75 % after 17 h for the three samples. Then, for the samples β and χ the light emission continued to progressively decrease with time and was extinguished within 48 or 58 h, while for sample α it remained stable up to 58 h after which it sharply decreased at 73 h. Variation of bioluminescence intensity did not show any relationship with day/night cycle for any of the samples (Fig. 2A).

The weight of the three samples decreased with time due to dehydration (Fig. 2B). The rate of decrease of weight was constant during the first 48 h of the experimental period. Once reaching weights < 0.48 g the samples had dramatically lower levels of bioluminescence (Fig. 2C).

At time 73 h in the day/night variation experiment the three samples of wood showed dim bioluminescence averaging 68, 65 and 63 RLU/s for samples α , β and χ , respectively. This level was about twice the control value of 15 – 35 RLU/s for tubes

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6 containing water only. Bioluminescence increased dramatically when water was
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8 added to the samples. Light emission measured 9 h after hydration had values of 1.1
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10 $\times 10^6$ RLU/s for sample A, an increase of 1,557,000 %; 2.8×10^4 RLU/s for sample
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12 B, an increase of 43,000 %; and 400 RLU/s for sample C, an increase of 600 % (Fig.
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15 3).

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18 The increase in bioluminescence intensity upon addition of water to wood
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20 samples was immediate (Fig. 4). Before addition, bioluminescence was about $1.7 \times$
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22 10^5 RLU/s; the addition of water was immediately followed by a doubling of light
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24 production in 1 min. The increase in emission continued for 5 min reaching values of
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26 5.9×10^5 RLU/s, and then leveled off for the remaining 24 min reaching values of
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28 7.0×10^5 RLU/s. Overall, there was about a 400 % increase of light emission
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30 following hydration of the sample. The increase of bioluminescence with time fit a
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32 logarithmic model (Fig. 4).
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37 Bioluminescence intensity was affected by temperature change and decreased
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39 >90 % when exposed for 15 min or more to -20 °C, 4 °C, and 60 °C (Fig. 5). At 15
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41 °C, bioluminescence decreased to 75 % of the initial light production after 15 min, 50
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43 % after 120 min, and 40 % after 360 min. At 37 °C, bioluminescence remained at
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45 about 100 % after 30 min before decreasing to 80 % after 60 min, 50 % after 120 min
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47 and to 10 % after 360 min. At the ambient temperature of 24 °C, bioluminescence
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49 remained essentially unchanged throughout the entire experimental period.
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DISCUSSION

This study is unique in reporting bioluminescence characteristics of a freshly collected luminous fungus on its natural wood substrate as opposed to fungus cultured in the laboratory on plates. The species of the fungus remains undescribed because the mycelium was the only part of the fungus found, making its taxonomic identification difficult. Thus it is not known whether the fruit bodies of the fungus also display bioluminescence, making the association with a specific taxonomic group speculative. The fluorescence emission spectrum peak at 480 nm may be related to light production (17) because all other peaks were chlorophyll related. In many luminescent organisms the fluorescence emission peak is similar to the bioluminescence emission peak (20, 21). If the 480 nm fluorescence emission peak from the fungus is similar to its bioluminescence emission peak, then the emission is unusually short-wavelength, because most fungi have bioluminescence emission spectra maxima between 520-530 nm (22). There are no published data on the fluorescence emission characteristics of other luminescent fungi.

This study shows that light production in the fungus is a continuous phenomenon that does not vary between day and night. This is in agreement with the study of Weitz et al. (2001) while it differs with others that suggest a diurnal periodicity in the light production intensity, though under experimental conditions (23-25).

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6 In terms of energy allocated to the bioluminescence reaction, this study
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8 supports the scenario where the light production is associated with low energy cost
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10 for the fungus. This would imply that the luminous reaction involves a by-product
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12 material or secondary metabolite, allowing the bioluminescence to be continuous (3).
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14 This is observed, for example, in luminous bacteria where reduced flavin
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16 mononucleotide (FMNH₂) is the substrate of the luminous reaction together with a
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18 long chain fatty aldehyde. The FMNH₂ is a by-product of the respiratory chain and is
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20 therefore continuously available for bioluminescence. Thus, in the bacterial system,
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22 the level of luciferase controls the amount of light produced (26, 27).
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27 Light production was sensitive to temperature and stayed maximal only at
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29 ambient temperature, as observed for other luminous fungi (10, 11). The change in
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31 bioluminescence may be related to changes in mycelial growth that shows similar
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33 sensitivity to temperature (10, 11).
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36 Bioluminescence involves an enzymatic reaction whose rate increases with
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38 temperature within levels that are ecologically realistic for the luminous organism.
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40 With this in mind, one would expect the 37 °C exposure tested in this study to trigger
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42 an increase of bioluminescence, at least initially. This was indeed observed when
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44 exposing cultures of the luminous fungus *Panus stipticus* to 33 °C and 37 °C; the
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46 bioluminescence first peaked within 5 to 7 min following the increase of
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48 temperature, then decreased gradually over time, returning to the initial level of
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50 bioluminescence 15 min after exposure (28). In this study the short-term (15 min)
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6 measurement of bioluminescence after exposure to higher temperature was certainly
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8 past any peak of light production that could have occurred due to temperature effect
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10 on the enzymatic reaction. Luminous fungi seem to react within minutes to changes
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12 in their environmental conditions and greater time resolution is therefore necessary to
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14 address their initial reaction.
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18 Following exposure to lower temperature (15 °C) the light production process
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20 was rapidly decreased within 15 min indicating that the enzymatic reaction
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22 associated with the production of the substrate and leading to the light production is
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24 slowed down and inhibited below the optimal condition of ambient temperature.
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26 Similar results have been observed for the bioluminescence from fungus fruit bodies
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28 (11).
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32 Light production in fungus is a continuous process that is inhibited by
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34 superoxide dismutase (SOD) present in high concentration in the fungus tissue (17,
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36 18). Thus according to this scenario, changing the activity of SOD could then change
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38 the level of produced light, yet without changing the level of substrate that seems to
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40 always be available and produced in live tissue. In plant cells temperature affects the
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42 concentration of reactive oxygen species and SOD activity (29, 30). In this study the
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44 decrease of bioluminescence after long exposure (6 h) to different temperatures was
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46 greater for higher temperature than for lower temperature, with 15 % and 50 %
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48 activity remaining, respectively. Assuming the involvement of a SOD regulation
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50 mechanism on bioluminescence in fungus, present data suggest that higher
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6 temperature induces greater levels of oxidative stress resulting in greater levels of
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8 SOD and thus greater inhibition of bioluminescence. This scenario would be
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10 complemented with other adverse denaturing effects of temperature affecting directly
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12 the molecular system leading to the light production.
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15 This study confirms prior results (19) that the bioluminescence of fungus
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17 increases upon exposure to water. From the chemical standpoint this observation
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19 supports the hypothesis that hydration dilutes the light-suppressant SOD, leading to
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21 an increase of bioluminescence (17, 18). Indeed, the increase of bioluminescence is
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23 so rapid following exposure to water that it is unlikely that the production of the
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25 substrate would be increased in such a short period of time. In addition, water may be
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27 a limiting factor controlling solubility of reagents involved in the fungus light
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29 production process. Indeed, the luminous substrate belongs to the sesquiterpene
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31 family and has low water solubility (16). This scenario is consistent with prior
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33 findings that full immersion of luminous mycelium on wood for a longer period (up
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35 to 376 h) increases light production intensity while decreasing the luminescence
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37 decay rate (19).
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43 Bioluminescence is typically considered a form of optical communication, in
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45 which the light emission is perceived by another organism that then changes its
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47 behavior. Yet in luminous fungus the function of its bioluminescence is unknown.
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49 One suggestion is that the light emission attracts animals that could disperse the
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51 spores more widely, yet this would only be valid for species with luminous fruit
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bodies (3, 4). The presence of luminous mycelium in many species argues against a putative function associated with bioluminescence in luminous fungus. Here, we suggest that bioluminescence of the mycelium has the function to attract animals that potentially carry fungal spores in well hydrated areas, as light emission from the mycelium is higher under hydrated conditions. Therefore spores would be deposited in an environment that is already optimal for growth. This is essential for the Basidiomycetes whose development is closely dependent upon water availability (11, 12). In species with a luminous mycelium, the mycelium would therefore have a dual function in performing the fungal translocation that permits transport of substances from further environment back to the fruit body (31, 32), and in attracting disseminating vectors towards environments favorable for development of the species.

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Figure caption
(Deheyn and Latz)

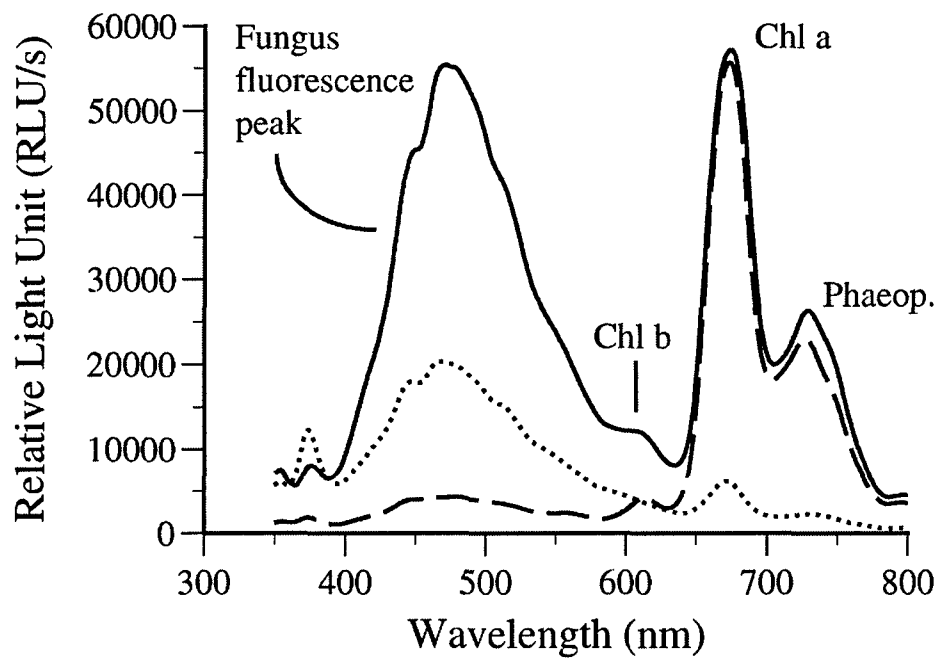
Figure 1: Fluorescence emission spectrum (excitation @ 380 nm) of outer wood sample with intense bioluminescence (black line), deeper wood sample with weak bioluminescence (dotted line), and outer wood sample with no bioluminescence (dashed line). Fluorescence from fungus peaks at 480 nm while peaks Chl_a, Chl_b, and Phaeop. correspond to chlorophyll *a*, *b*, and Phaeophytin pigments, respectively.

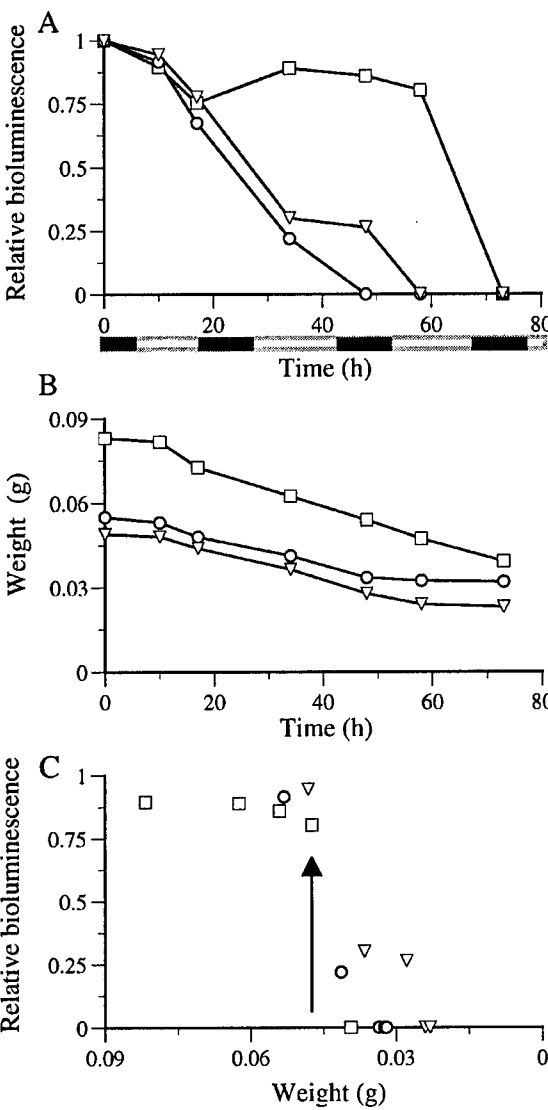
Figure 2: Temporal change for three samples α (square), β (circle), and χ (triangle) of mycelium on wood. (A) Spontaneous bioluminescence; the black and white bars specify the time of night and day, respectively. (B) Change in weight showed a linear decrease over the time of the experiment. (C) Change of bioluminescence intensity with weight. The arrow corresponds to a weight of 0.046 g below which any change in weight was accompanied by a large decrease in bioluminescence.

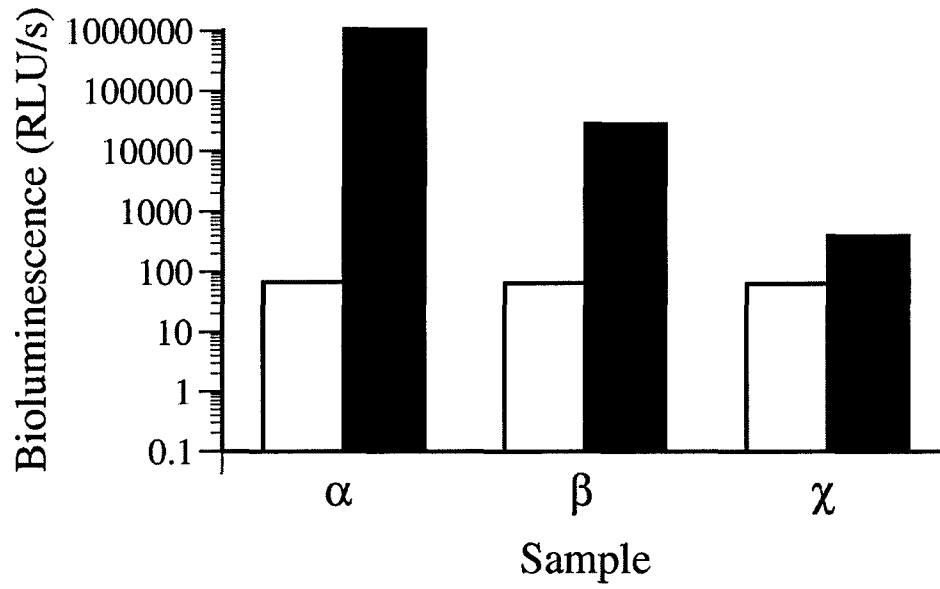
Figure 3: Long-term change in bioluminescence intensity after hydration of the three samples α , β , and χ of mycelium on their wood substrate. Bioluminescence measured 9 h after hydration (black bar) was much greater than that prior to addition of water (white bar).

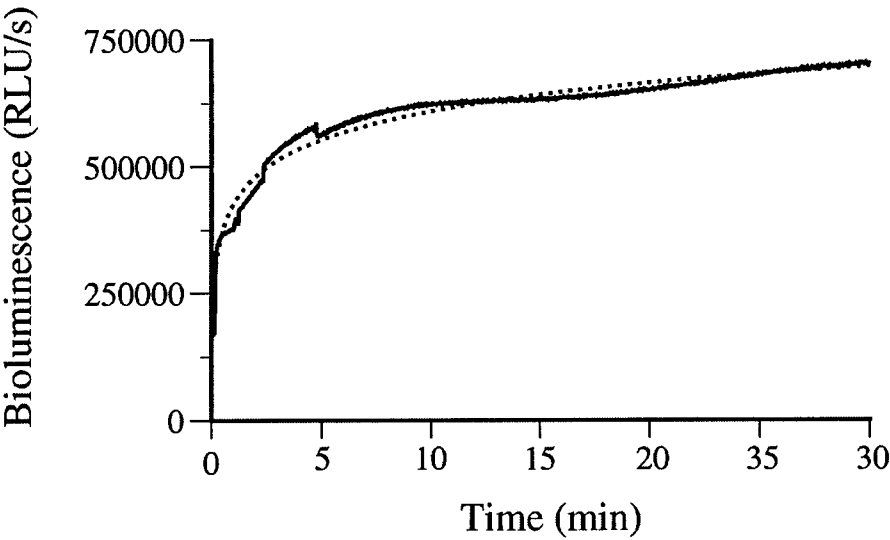
Figure 4: Short term variation in bioluminescence intensity after hydration of a sample of mycelium on wood. The bioluminescence increased about 400 % from initial values. The data (in black) are modeled with the logarithmic expression (in dotted line): $y = 80232 * \ln(x) + 95633$ ($R^2=0.96$).

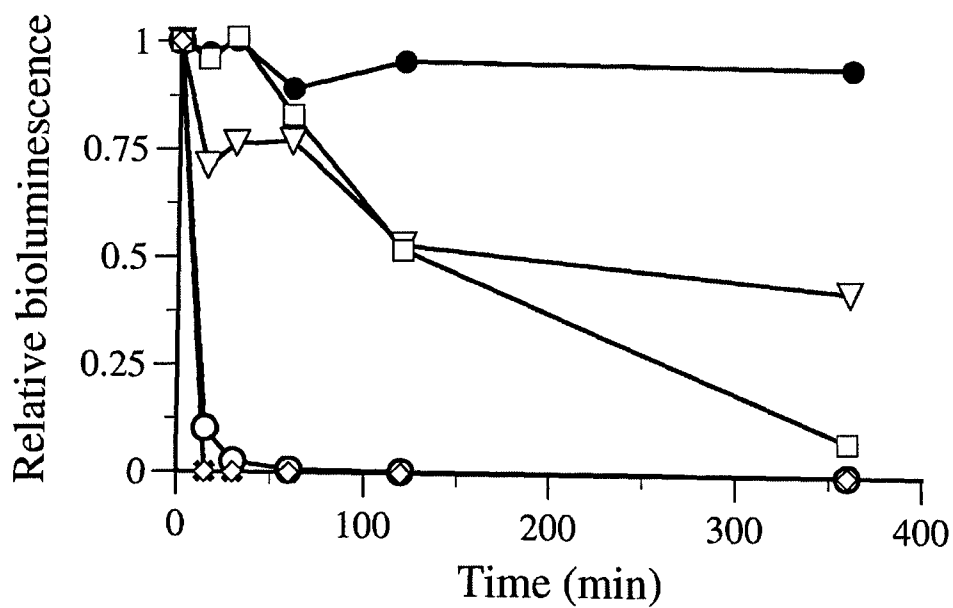
Figure 5: Variation of bioluminescence over a 360 min exposure to various temperatures. For samples at -20°C (cross), 4°C (open circle) and 60°C (diamond), bioluminescence was extinguished within 15 min. Bioluminescence of samples at 15°C (triangle) and 37°C (square) decreased over time while that for samples at 24°C (filled circle) remained unchanged during the experimental period.











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Abstract: Green fluorescent proteins (GFPs) are well known for their intensive use in cellular and molecular biology that take advantage of the GFPs self-folding and built-in fluorophore characteristics as biomarker. Occurrence and function of GFPs in Nature is less known. For a long time GFPs were described only from some cnidarians, and it is only recently that it was also found in copepod crustaceans. Here we describe a GFP from *amphioxus* (Chordata: Cephalochordata). This is the first time an endogenous GFP has been found in any representatives of the deuterostome branch of the Animal Kingdom. We have isolated and characterized a gene (AmphiGFP) from *Branchiostoma floridae* that encodes a GFP protein related to those of cnidarians and copepods in both its amino acid sequence and also its predicted higher order structure (an 11-stranded β -barrel enclosing a fluorophore). Phylogenetic analyses with Bayesian and maximum parsimony demonstrate that the AmphiGFP protein is markedly more closely related to copepod than to cnidarian GFPs. In adults of all three *amphioxus* species, the green fluorescence is strikingly concentrated

anteriorly. The anterior end is the only body part exposed to light in these shallow-water dwellers, suggesting possible photoreceptive and/or photoprotective functions for the endogenous GFP.

Endogenous Green Fluorescent Protein (GFP) in Amphioxus

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Running head: Amphioxus Green Fluorescent Protein

Green fluorescent proteins (GFPs) are well known for their intensive use in cellular and molecular biology that take advantage of the GFPs self-folding and built-in fluorophore characteristics as biomarker. Occurrence and function of GFPs in Nature is less known. For a long time GFPs were described only from some cnidarians, and it is only recently that it was also found in copepod crustaceans. Here we describe a GFP from amphioxus (Chordata: Cephalochordata). This is the first time an endogenous GFP has been found in any representatives of the deuterostome branch of the Animal Kingdom. We have isolated and characterized a gene (AmphiGFP) from *Branchiostoma floridae* that encodes a GFP protein related to those of cnidarians and copepods in both its amino acid sequence and also its predicted higher order structure (an 11-stranded β -barrel enclosing a fluorophore). Phylogenetic analyses with Bayesian and maximum parsimony demonstrate that the AmphiGFP protein is markedly more closely related to copepod than to cnidarian GFPs. In adults of all three amphioxus species, the green fluorescence is strikingly concentrated anteriorly. The anterior end is the only body part exposed to light in these shallow-water dwellers, suggesting possible photoreceptive and/or photoprotective functions for the endogenous GFP.

Green Fluorescent Proteins (GFPs) are familiar to most biologists as invaluable tools for cellular and molecular biology (1). However, in spite of the considerable effort spent on developing GFPs for laboratory reagents, much remains to be learned about the taxonomic distribution and biological function of these proteins in Nature. To date, GFPs have been found in only two major groups in the metazoan tree: specifically, in a number of cnidarians, relatively near the base of the tree, and in a few copepod crustaceans, relatively derived within the protostome branch (2, 3). The cnidarian GFPs are often associated with bioluminescence, but those found so far in copepods are not. We now report that the limited taxonomic distribution of animals with endogenous GFPs may be partially due to inadequate sampling efforts, because we have found such molecules in the cephalochordate amphioxus. About ten years ago, we began to suspect that endogenous GFPs are present in amphioxus, because the eggs and embryos emit a uniform green fluorescence when illuminated with UV light (this phenomenon is illustrated in reference 4). The present note reports on the isolation and molecular characterization of indubitable GFPs from three amphioxus species (none of which are bioluminescent). This is the first demonstration of presence of these distinctive molecules in any deuterostome. In addition, the tissue distribution of amphioxus fluorescence, interestingly localized at the anterior end of the adult body, gives insights into possible functions of the endogenous GFPs (discussed below).

The present note concerns three amphioxus species: *Branchiostoma floridae* Hubbs, 1922 (the Florida amphioxus, collected in Tampa, Florida), *Branchiostoma lanceolatum* (Pallas, 1774) (the European amphioxus, collected in Banyuls-sur-mer, France), and *Branchiostoma belcheri* Gray, 1847 (the Asian amphioxus, collected in Misaki, Japan). For adults of these three species, the fluorescence spectra, stimulated by incident UV (380 nm), had peaks at 524 nm, 526 nm, and 527 nm, respectively (Fig. 1). To establish a link between this green fluorescence and a possible endogenous GFP in the tissues of amphioxus, a cnidarian protein (GenBank AY157666) was blasted (tblastn) using the EST_OTHERS database in GenBank. The search identified

numerous clones of GFP from unfertilized eggs, gastrula, neurula, larval, and adult libraries for *B. floridae*, all sharing the same sequence that was yet distinct from the cnidarian sequence. Using primers based on the EST nucleotide sequence, we obtained cDNA of the expressed gene by reverse transcriptase RT-PCR with mRNA extracted from the adult *B. floridae* as the template. The cDNA sequence is identical to the EST sequence. The sequence, which we named *AmphiGFP* and deposited in GenBank (EF157660), encodes a protein of 218 amino acids. Conversion of the amino acid sequence of AmphiGFP to a three-dimensional structure by Swiss-Model (ExPASy server) and modeling from the known crystal structure of a fluorescent protein from a copepod (5) showed that the predicted higher order structure of the amphioxus protein closely resembles that of endogenous fluorescent proteins in cnidarians and copepods. All these molecules comprise an 11-stranded β -barrel enclosing a central strand that includes a cyclized tripeptide fluorophore—based on glycine-tyrosine-glycine in both amphioxus and copepods, but on serine-tyrosine-glycine in most fluorescent cnidarians.

We used UV irradiation (380 nm) to study the tissue distribution of the green fluorescence in living developmental stages and adults of the Florida amphioxus, in living adults of the European amphioxus, and in RNA/*ater*-preserved adults of the Asian amphioxus (Fig. 2). In the Florida species, the fluorescence, which is ubiquitous in the eggs and larvae (4), first becomes patchily distributed in the larvae (Fig. 2A, B), and finally becomes localized at the anterior end of the juveniles and adults, exclusively in the support cells of the oral cirri (although not in the skeletal arch from which they spring) (Fig. 2C, D). In ripening adults of the Florida amphioxus, fluorescence is also detected in the ovaries, specifically in the growing oocytes, but not in the testes (data not shown). In adults of the European amphioxus, UV also induces green fluorescence intensely in the oral cirri and more diffusely in the epidermis—chiefly at the anterior end of the body, but also very inconspicuously near the posterior end (Fig. 2E, F); the anterior fluorescence is in the support cells of the cirri, but not in the skeletal arch (Fig. 2G). Adults of the Asian amphioxus also emit green fluorescence—strongly from the

supporting cells of the oral cirri and their skeletal arch support (Fig. 2H); as well as weakly from the epidermis at the anterior end of the animal.

AmphiGFP belongs to the 11-stranded β -barrel superfamily of proteins. This superfamily is considered to include some proteins that fluoresce in colors other than green, some chromoproteins that do not fluoresce at all, and G2FP motif proteins, which are components of the extracellular matrix of most metazoans (6-9). We assessed the relationships between AmphiGFP and other known proteins in the superfamily by phylogenetic analyses with Bayesian analysis and maximum parsimony (Fig. 3). In our analysis, amphioxus GFP is more closely related to fluorescent proteins of copepods than to those of cnidarians, an arrangement in accord with current and previous hypotheses of metazoan phylogeny (10). A fair indication of the degree of similarity among fluorescent proteins of these three animal groups is indicated by only 19% amino acid identities between AmphiGFP and a cnidarian (*Aequorea victoria*) GFP as contrasted to 35% amino acid identities between AmphiGFP and a copepod (*Pontellina plumata* ppluGFP2) GFP. Importantly, our phylogenetic analysis is consistent with earlier work (2) suggesting that fluorescent proteins of bilaterian animals originated from a single ancestral fluorescent protein in a basal metazoan. Thus, AmphiGFP is evidently not independently derived from a deuterostome G2FP motif protein (several of which are included in our analysis).

The ecological significance of endogenous fluorescent proteins is poorly understood (2, 11, 12). Even so, the tissue distribution of AmphiGFP suggests a couple of possible functions for the endogenous molecule in amphioxus. One is photoreception, as indicated by the localization of fluorescence in support cells of the oral cirri, although these structures are not one of the four currently recognized photoreceptive tissues in amphioxus (13). There is, however, ultrastructural evidence consistent with the possible role of oral cirri in photoreception: cirral support cells stack on top of each other delimiting in-between cells extracellular pockets in which project microvilli and cilia (one per cell) (14, see Fig. 89), the whole stack of cells being associated with axonal

processes of presumed sensory neurons (15). These characteristics are the ones of rhabdomeric photoreceptor organs (16, 17), which activity could be coupled with AmphiGFP knowing that, like other fluorescent proteins, AmphiGFP can probably undergo reversible photochemical transformations (18) and thus have the potential to transduce light energy into chemical energy. The photoreceptive function is also suggested when considering the ecological behavior of the animal that lives burrowed in the sand except for its head from which oral cirri face the water column and thus the downwelling sunlight. The animal in this almost completely burrowed position has long been shown to be sensitive to change in light exposure (19).

A second possible function AmphiGFP may be protection against UV. This is suggested by the presence of fluorescence throughout the tissues of the pelagic amphioxus embryos as well as the concentration of fluorescence at the anterior end of the body of the adult—the end that usually projects slightly from the burrow of these relatively shallow living marine animals. At a first level of defense, the aromatic fluorophore core of AmphiGFP could absorb UV and dissipate it as less damaging, lower energy fluorescence (12, 20). Moreover, at a second level of defense, the molecule could act as an anti-oxidant to detoxify reactive oxygen/free radicals (21), because imidazolinone fluorophores are known to have a high affinity for molecular oxygen (22-24).

In conclusion, with our demonstration of AmphiGFP, amphioxus becomes the only deuterostome known to contain an endogenous fluorescent protein. Even with this discovery, the distribution of fluorescent proteins among animals remains sparse and widely scattered—with known representatives in only one isolated group of deuterostomes (amphioxus), in one isolated group of protostomes (a few copepods), and in one group of relatively basal metazoans (namely, some hydrozoan and anthozoan cnidarians). This sparse distribution could be indicative of horizontal gene transfer, although there are not many well accepted examples of this phenomenon in metazoans (25, 26), or from secondary loss from most taxa, or from inadequate

taxonomic sampling. It is possible that more members of the highly distinctive 11-stranded β -barrel protein superfamily (other than the ubiquitous G2FP proteins) remain to be discovered, and some of these, not necessarily fluorescent, might be relatively common and involved in functions more general than the production of conspicuous fluorescence.

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Figure Captions

Figure 1 Fluorescence spectra (with emission maxima) from intact adult amphioxus illuminated with UV (380 nm) and measured by SE200 low light digital spectrograph (Catalina Scientific Instruments, Tucson, AZ). Spectra from living specimens of Florida and European species, and from a specimen of the Asian species preserved in *RNA/later* (Ambion, Austin, TX) (exposure to *RNA/later* does not alter GFP emission spectra; our unpublished observations). For comparison, emission spectrum for EGFP from hydrozoan cnidarian *Aequorea* (Biovision Research Products, Mountain View, CA) measured in same apparatus is included.

Figure 2 Fluorescence of intact specimens of three amphioxus species under UV illumination (380 nm). (A) Early larva of Florida species under visible light; (B) same under UV with patchy green fluorescence, most intense just posterior to the mouth (arrow). (C) Postmetamorphic juvenile of Florida species under visible light, with oral cirri indicated by arrow; inset, oral cirri of same animal fluorescing under UV. (D) Detail of fluorescing support cells of Florida species in oral cirri of adult under UV (arrow indicates that no cells of skeletal arch fluoresce). (E) Adult specimen of European species under visible light, with oral cirri indicated by arrow. (F) Same animal under UV with fluorescence conspicuous anteriorly and weak posteriorly (arrow). (G) Detail of oral cirri of adult European amphioxus (arrow indicates no cells in skeletal arch fluoresce). (H) Detail of oral cirri of adult Asian amphioxus (arrow indicates fluorescence in skeletal

arch). Scale line in top left panel is: 100 μm for A and B; 250 μm for D; 300 μm for C, G, and H; and 3 mm for E and F.

Figure 3. Phylogenetic analysis of the 11-stranded β -barrel protein superfamily in animals. Sequences for AmphGFP and a recently discovered copepod fluorescent protein from *Chiridius poppei* (9) were aligned, using ClustalW (27) and manual adjustment, against the cDNA dataset and alignment from a previous analysis (2), which includes a range of fluorescent proteins from Cnidaria and copepod arthropods and for G2FP motif proteins from three deuterostomes (tunicate, mouse, and human). The alignment used and results are available at Treebase (www.treebase.org). Maximum parsimony analyses of the 816 characters (696 parsimony informative) were performed using PAUP* 40b10 (28), with default settings, except for stepwise addition using 100 random addition sequences. This resulted in a single shortest tree of length 5335. Bootstrap and Jackknife (with 37% deletion of characters) values were assessed in PAUP* using 1000 replicates, and the majority rule consensus tree matched the most parsimonious tree. Bayesian analyses with MrBayes 3.1 (29) were run with default priors (rate matrix: 0-100, branch lengths: 0-10, Gamma shape: 0-1), a random starting tree and six Markov chains. MrModelTest 2.2 (30) indicated use of the GTR+I+G model under both the hLRT and AIC criteria. 1,100,000 generations were run with a tree saved every 1000 generations. The first 100,000 generations (100 trees) were discarded as burn-in. The majority rule consensus tree of the remaining 1000 trees for each analysis gave the posterior probabilities for each clade. Both Maximum parsimony and Bayesian analyses gave congruent results and show that AmphGFP is most closely related to its homologs in copepod arthropods (protostomes) and is not derived independently from non-fluorescent G2FP proteins. Bayesian posterior probability values to the left of supported nodes; Bootstrap (bold) and Parsimony Jackknife (italics) values to the right (values less than 90% not shown).

Figure 1
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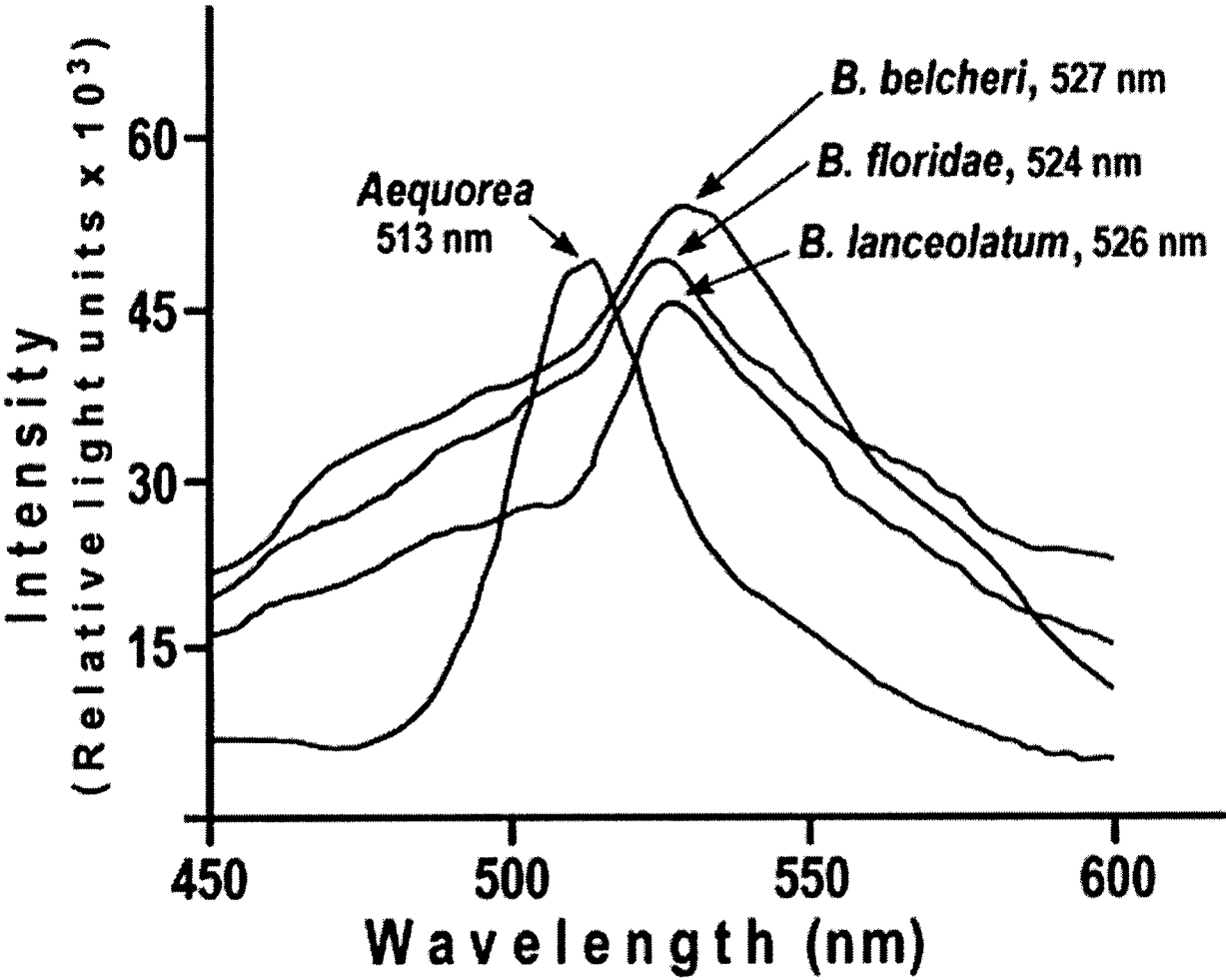


Figure 2
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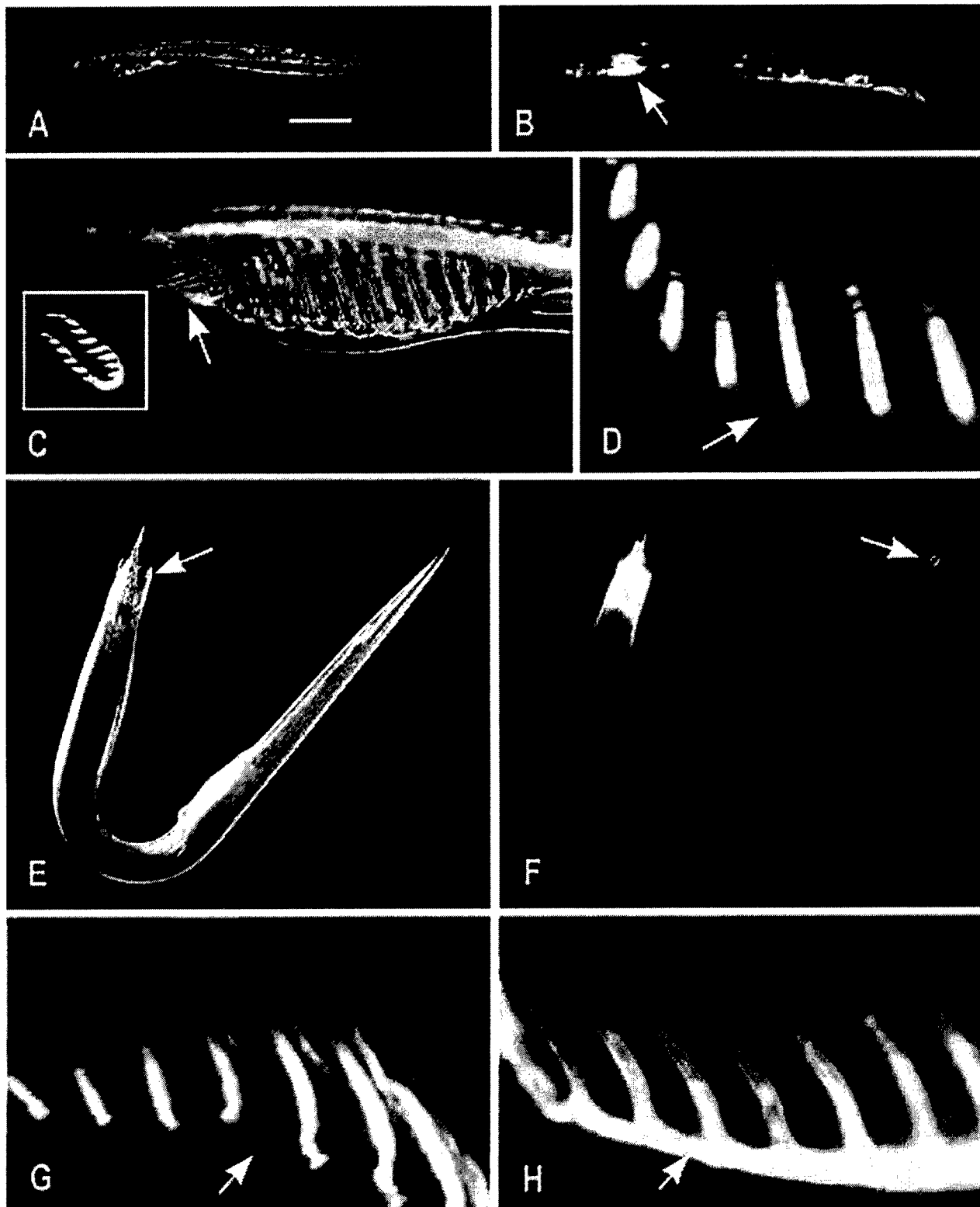
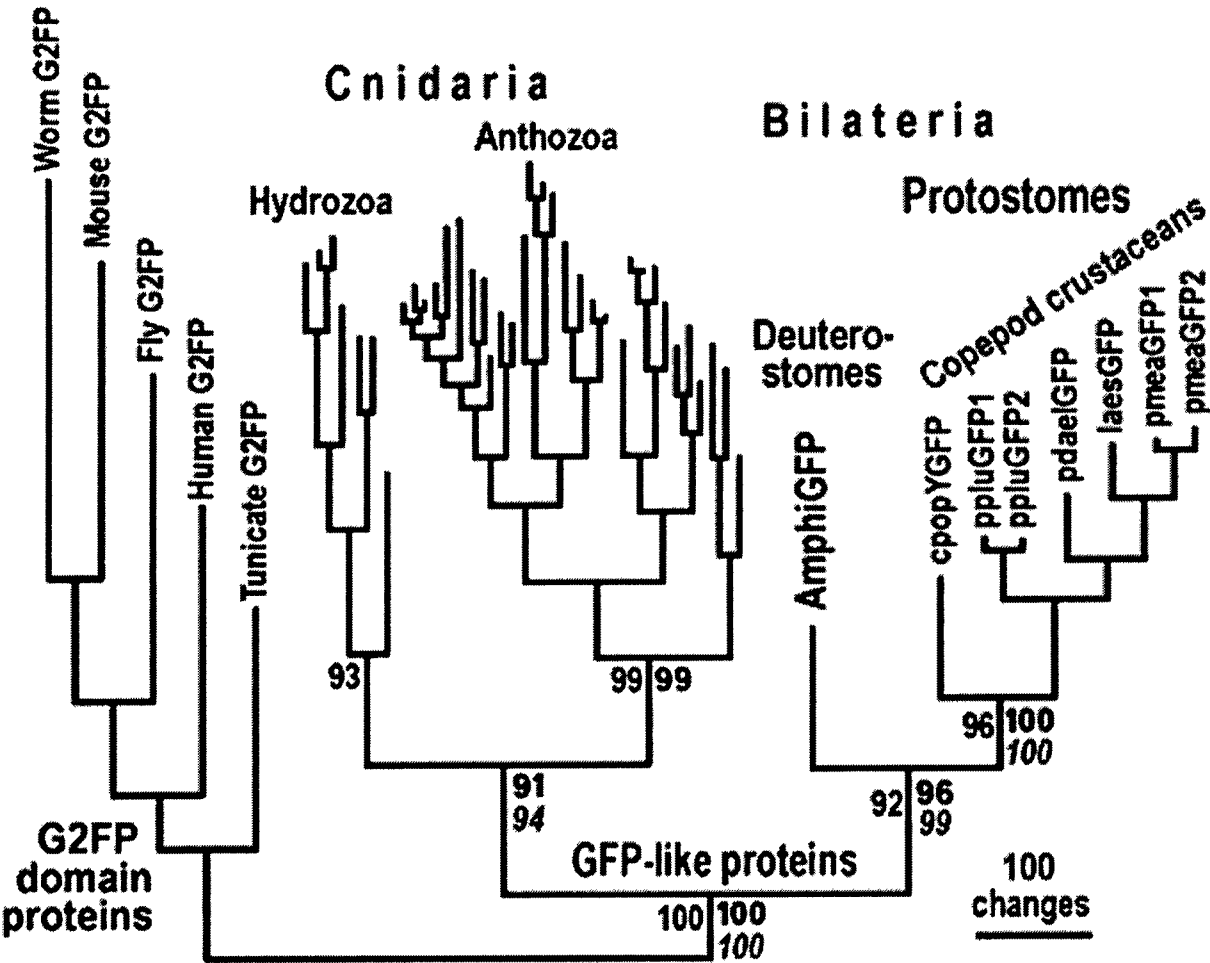


Figure 3
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**Bioluminescence characteristics of the marine
polychaete *Odontosyllis phosphorea* (Syllidae)**

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Abstract

The syllid polychaete *Odontosyllis phosphorea* is known to produce brilliant displays of bioluminescence during mating swarms. Freshly collected individuals were studied in the laboratory to understand the characteristics of its luminescent system. Light emission appeared as an intense glow after stimulation with potassium chloride, associated with the secretion of mucus. The mucus was viscous, blue colored, and exhibited long-lasting glowing that was greatly intensified by addition of peroxidase or ammonium persulfate. The emission spectrum of bioluminescence was unimodal with a maximum spectral emission between 494-504 nm depending on the mode of chemical stimulation. The fluorescence emission spectrum was similar, yet always of lower intensity unless it originated from mucus that already had produced light, indicating that the oxidized product of the light production is the source of fluorescence. Bioluminescence was produced from juveniles as small as 0.5-1.0 mm, in which case the light remained mainly internal and was not secreted as mucus. This early occurrence of bioluminescence in the life cycle of *O. phosphorea* indicates that bioluminescence is innate to the post-metamorphic stage of the worm and used for purposes other than attracting mates. The mixing of hot and cold extracts of the mucus did not result in light emission, and mucus samples exposed to oxygen depletion by bubbling with argon or nitrogen were still able to produce intense bioluminescence. The luminous system was functional at temperatures as low as -20°C and was degraded above 40°C . These results suggest that bioluminescence involves a photoprotein and not a luciferin-luciferase reaction.

Keywords: Bioluminescence; Emission spectrum; Ecological function; Fluorescence; Mucus; Photoprotein

1. Introduction

Many marine organisms produce bioluminescence, light emission associated with intra- and/or inter-specific signals of communication for attracting mates or prey, or for defense against predation (Herring 1978; Morin 1983). For the communication signal to be effective, the light should transmit well through ocean water and be detected by the appropriate receiver. In general, the maximum emission of bioluminescence occurs in the blue-green region of the visible spectrum (460-490 nm) for open-ocean pelagic organisms, and has maximum emission in the green (480-510 nm) for organisms living in benthic or shallow coastal environments. Bioluminescence also has different intensities, colors and temporal patterns (flash versus glow) depending on the species and the function associated with the light signal (Morin 1983).

The marine worms of the genus *Odontosyllis* have long been known for their bioluminescence displays (Galloway and Welch 1911; Crawshaw 1935). The worms, called palolo worms in the South Pacific, are reported at certain times of the lunar cycle to swim at the sea surface where they produce intense bioluminescence (Markert *et al.* 1961; Tsuji and Hill 1983). The light is released as a puff of glowing material in the water that, when thousands of individuals are swarming, turns into an extensive luminous cloud (Crawshaw 1935).

The worms inhabit shallow coastal areas and are normally benthic. Swarming is initiated by females that swim to the sea surface where they secrete luminous mucus and eventually release their gametes through epitoky. The bright bioluminescence attracts males that swim from the seafloor and release their gametes in the luminous cloud, also through epitoky. Males also produce bioluminescence when approaching the luminous cloud, but the light production then can be either secreted from the body as for females, or remain internal, occurring as a series of short flashes produced from the sagittal area of the male posterior section. Such a pattern is also

observed in females that are mechanically stimulated when located on the seafloor, suggesting that the light production in *Odontosyllis* is used as a visual signal to attract mates but also as a startle mechanism against predation during nocturnal foraging (Fischer and Fischer 1995).

Although popular for its bioluminescence display, the bioluminescence of *Odontosyllis* has been little studied because of practical difficulties in obtaining samples in quantities sufficiently large and pure for optical and/or biochemical investigations. The worms are relatively small in size and difficult to catch in their benthic habitat where they are cryptic and live inside silk-like tubes during the day. The worms are primarily observed in the field by virtue of their bright bioluminescence released during swarming nights. However, by that time the worms have released most of their luminous material and thus are less appropriate for studying their bioluminescence. The other feasible approach, collecting samples of the luminous water, is also not effective because the samples contain gametes and exhausted and dilute luminous material, in addition to microorganisms and various other substances found in seawater (Fischer and Fischer 1995). Clearly the lack of high throughput sampling methodology has been a significant limitation on carrying out research on the bioluminescence of *Odontosyllis*.

The biochemistry of light production in *Odontosyllis* is thought due to a luciferin-luciferase reaction based on hot/cold extracts from worms (Harvey 1952). Based on collected material from >50,000 worms of *O. enopla*, bioluminescence has an emission maximum at 507 nm following an oxidation process, is sensitive to pH and optimal at seawater pH 7-8, and uses cyanide and/or magnesium as a co-factor (Shimomura *et al.* 1963; Shimomura *et al.* 1964).

This study investigates the bioluminescence system of freshly collected *Odontosyllis phosphorea* and is unique in studying internal and secreted light emission from individual

worms. Bioluminescence was chemically stimulated and persisted under oxygen-depleted conditions, suggesting that it involves a photoprotein.

2. Materials and Methods

Odontosyllis phosphorea swarms during the summer in the 2 m-shallow area of De Anza Cove, Mission Bay, located in San Diego, California (Tsuji and Hill 1983). In this study, after verifying that the luminous behavior was still occurring at nighttime in the cove, individuals were collected during daytime by sampling the material covering the seafloor bottom of the cove. Samples were obtained between May and October in 2004 and 2005, and consisted of the algae *Ulva* sp. and bryozoan *Zoobotryon verticillatum*, which were kept in jars with seawater for transport to the nearby research laboratory at Scripps Institution of Oceanography. There, samples were distributed into different containers to allow small organisms using the algae and bryozoan as protective substrate to escape and swim into the water. This behavior was facilitated by gently swirling the substrate, sometimes after short-time exposure (10 min) to cold temperature (4 °C), which anesthetized the organisms and facilitated their separation from the substrate into the water. This technique yielded many small invertebrates, including copepods, pycnogonids, and polychaetes. On a good collection day, dozens of specimens of *O. phosphorea* of various sizes, sexes, and maturity stage would be seen crawling on the walls of the containers, sometimes starting to build their silt-like tubes around them (Fischer and Fischer 1995). The worms were then individually collected using a microprobe or disposable Pasteur pipette, and placed into a container filled with artificial seawater (ASW; Deheyn *et al.* 1997). Worms were maintained at room temperature until used in experiments. Some worms were maintained for

long periods in an aquarium containing aerated sand-filtered natural seawater that was changed every other week. On a monthly basis, 3 worms were removed and tested for bioluminescence using KCl stimulation.

Many compounds involved in bioluminescence are also known to be fluorescent. Worms of small size lack the dark-brown pigmentation of adults and were photographed using epifluorescence microscopy (Zeiss Axioskop with Spot mercury lamp, Diagnostic Instrument Inc.) to identify the distribution of the potential areas of bioluminescence.

Stimulation of bioluminescence. Individuals of *O. phosphorea* never showed spontaneous bioluminescence yet light was always produced following mechanical or chemical stimulation from larger individuals. Light production was then triggered by exposure to potassium chloride (KCl, 200 mM final concentration), which is known to depolarize cell membranes and stimulate bioluminescence in other luminous invertebrates (Germain and Anctil 1988; Deheyn *et al.* 1997). In *O. phosphorea*, light production can both internal and secreted from the body. Accordingly, the KCl treatment induced secretion of a luminous mucus, which was accompanied by frenetic swimming and sometimes epitoky when collecting the worms close to the swarming periods.

The bioluminescence from the mucus was expressed as a long-lasting glow whose intensity was further increased by addition of 0.04 % (v:v) peroxidase or 20 mM ammonium persulfate ($(\text{NH}_4)_2\text{S}_2\text{O}_8$) (APS, Sigma). Working with the luminous mucus thus presented a unique opportunity for assessing optical and biochemical characteristics of on-going processes involved in the light production of *O. phosphorea* under controlled laboratory conditions. In this study, all experiments assessing the possible effect of a biochemical treatment on light production were conducted using luminous mucus. Mucus samples were obtained by treating 40 worms in a tube

containing ASW with 400 mM (v:v) KCl. The mucus was retrieved after several minutes following gentle centrifugation of the tubes. The resulting sample of luminous mucus was then subdivided according to the experimental plan. Following treatment, the residual bioluminescence capacity of the mucus samples was measured following treatment with peroxidase. Bioluminescence was recorded using a Sirius luminometer (Berthold Detection Systems) and expressed as Relative Light Units produced per unit of time (RLU s⁻¹). In some cases a 2.0 neutral density filter was used to avoid saturation of the luminometer, in which case the light intensity values were multiplied by 100 before data processing.

Emission spectrum of bioluminescence and fluorescence. Emission spectra were measured using a Low Light Coupled Intensified SE200 Digital Spectrograph (Catalina Scientific, Tucson AZ). This instrument uses a low-light charge coupled device (CCD) detector to measure the full 300-700 nm spectrum of light in one exposure, with no scanning necessary and thus with no alteration of the spectrum when light intensity varies during the recording process. The measurements were made using exposures ≥ 0.2 s, depending on the light intensity and gain of the intensifier, resulting in a spectral resolution of about 1 nm. Spectra were smoothed using the built-in smooth function, which applies a triangle-function convolution filter to the spectrum, with settings of 1,000,000 counts, 5 pixels, 20 nm, and 40 cm⁻¹.

Bioluminescence emission spectra were measured from adult individuals after KCl stimulation, and from luminous mucus stimulated using peroxidase or APS. Analyses were done separately between smaller and larger adult individuals to distinguish between genders {Fischer, 1995 #2024}. This size differentiation is based on sampling of worms during the reproductive period (May through September in De Anza cove, San Diego; {Tsuji, 1983 #689}). Smaller

individuals that were 5 to 8 mm long and lightly pigmented were considered to be males, while larger individuals of 12 to 15 mm long and heavily pigmented were considered to be females. Fluorescence emission spectra excited at 380 nm were measured from freshly KCl-stimulated mucus, and from mucus treated with peroxidase or APS. Exposure time and gain conditions for fluorescence measurements were optimized so that no bioluminescence was detected and thus the measured light signal was predominantly due to fluorescence. Controls for the bioluminescence and fluorescence measurements consisted of ASW blanks to which KCl, peroxidase, or APS was added.

Variation of bioluminescence with juvenile size. Twenty four specimens of small size, ranging from 0.5 to 2.5 mm body length, were considered for bioluminescence testing after KCl stimulation. Light emission was integrated over 2 min after which the specimen was removed from the tube using a microprobe and the bioluminescence of the remaining media (ASW + KCl \pm mucus) recorded again for 2 min, with no additional chemical stimulation. This protocol assessed whether light production was similar among individuals and differentiated bioluminescence due to internal production vs. secreted mucus. Variation of bioluminescence intensity with worm size was expressed for individual worms versus their corresponding mucus secretion, and the allometric variations modeled by a Gompertz-like equation {Deheyn, 1999 #9}:

$$B = B_{\infty} * e^{-b * e(-k * S)}$$

where B is the bioluminescence of an individual worm for a given size S, B_{∞} the asymptotic value of bioluminescence, b the ratio B_{∞}/B_0 in which B_0 is the bioluminescence of the smallest worm, and k the variation coefficient of the allometric model. The equation parameters were

estimated from the original values of bioluminescence using the nonlinear estimation method of Quasi-Newton, with robustness and significance of the model relationship indicated by R^2 and P values, respectively {Zar, 1996 #1774}.

Effect of temperature on bioluminescence. Samples of luminous mucus were exposed to -20, 4, 20, 40, 60, and 80 °C for up to 30 min. A total volume of 350 µl was exposed at each temperature from which a sub-sample of 50 µl was used for light measurement every 5 min. All samples were from the same stock of mucus and processed in parallel. Each bioluminescence measurement included three 10 s consecutive recording of light production kinetics from the same sample. Therefore, the first measurement represented light production from mucus, then from mucus + APS, and finally from mucus + APS + peroxidase. Controls that consisted of ASW ± APS ± peroxidase did not produce light. Mucus samples at -20 °C never reached the frozen state while samples at 80 °C showed some evaporation.

Decay of bioluminescence over time was modeled using a simple negative exponential model, $y = \alpha * e^{-\beta x}$, where α is the initial value of light intensity and β the slope, which represents the rate at which bioluminescence decreases over time under the various conditions of temperature: the greater the β value the faster the decay. Coefficient of determination R^2 of each model was tested for statistical significance by comparison with critical values {Zar, 1996 #1774}.

Hot/cold extracts of bioluminescence. Samples of luminous mucus were kept at room temperature (cold extract) or heated to 90 °C (hot extract) until bioluminescence decreased to <1

% of the original level. This decrease in bioluminescence took up to 90 min for cold extracts and 10-20 min for hot extracts. The hot extract was then left on ice until the cold extract was ready for use, and re-checked for lack of spontaneous bioluminescence before starting the hot-cold cross-reactivity experiment. Samples were made in triplicates so that the extracts could be combined as follows: hot + cold, hot + hot, cold + cold, hot + ASW, cold + ASW. Bioluminescence was measured for each combination of extracts and ASW for 1 min after addition of peroxidase. The experiment was repeated several times using mucus from different numbers of worms, and worms of different sizes.

Stimulation of bioluminescence under anoxic conditions. 300 μ l samples of fresh luminous mucus or individual worms in ASW were bubbled with argon or nitrogen for 10 to 30 min to deplete oxygen. The vials were then sealed with rubber stoppers and immediately measured for spontaneous bioluminescence for 1 min. A 300 μ l volume of KCl for worm samples, or APS for mucus samples, was then injected through the rubber stopper and bioluminescence recorded for an additional 1 min. Stock solutions of 20 mM APS and 400 mM KCl were bubbled with argon or nitrogen for 30 min before use. Controls consisted in bubbling the samples and solutions with air, and in analyzing non-bubbled samples. The experiment was repeated several times using mucus from different numbers of worms, and worms individuals of different sizes > 1mm, with always only one single specimen per experimental condition.

3. Results and discussion

For the first time the bioluminescence of *Odontosyllis phosphorea* was triggered chemically under experimental conditions in the laboratory. Bioluminescence appeared

immediately after KCl stimulation, peaking within seconds before decreasing as a long-lasting glow that could last for minutes. Subsequent addition of APS or peroxidase triggered an immediate increase to a plateau level where light emission persisted for >60 min depending on the size of the tested individual. Bioluminescence from both smaller (considered to be males) and larger (female) individuals generally followed this pattern with the intensity of produced light reaching similar values for both males and females (ranging from 10^8 to 10^{10} RLU s^{-1}). The kinetics associated with the KCl-peak of bioluminescence was different between genders, the peak appearing later and being broader in males (Fig. 1). In females, the light intensity always increased rapidly after stimulation, peaking about 1-3 s after KCl injection, followed by a rapid exponential decrease. About 60-70 s in the decay phase, the light intensity reached a steady level with an intensity about 30 times greater than prior to stimulation. Addition of peroxidase then triggered an immediate increase to 20 times greater intensity. Males had a similar kinetics profile except for variability of the KCl peak, which exhibited a maximum similar to that of females but then showed a second long-lasting peak during the exponential decay phase, or it showed only one peak that was reached later (10-20 s after stimulation) and lasted longer (30-50 s of relatively steady light intensity) (Fig. 1). These observations were based only on a total of 6 individuals (3 of each gender), which was not sufficient to carry out further analysis for statistical significance. Further study should consider a greater number of individuals from various stages of adult development and maturity in relation to the spawning period. In this study all tested females had mature ovaries whereas the state of maturity could not be ascertained for the smaller sized males. All tested individuals, however, appeared to be mature considering that the pattern of bioluminescence in smaller juvenile individuals was distinct, peaking several minutes after KCl stimulation and showing no significant increase of light emission with repeat stimulation with

APS or peroxidase (data not shown).

Multiple laboratory observations suggested that in larger individuals the bioluminescence stimulated with KCl was accompanied with the secretion of mucus, to an extent that varied with size and gender of individual, and with the period of the reproductive cycle; the closer to the swarming period the more mucus was produced. The mucus was highly viscous, in particular when stimulating several worms in a confined volume. Then the mucus would develop as threads that would stick to the worms and walls of the vials (Fig. 2A), making it difficult to collect the mucus separately from the worms. The high viscosity of the mucus suggests that it contains high concentrations of carbohydrates and/or has a high degree of sulfation (Takano 2002; Suzuki *et al.* 2003). There are no reports of sulfated polysaccharides involved in the bioluminescence reaction; the most similar is the luminous reaction of bacteria that involves aldehyde (Tu and Mager 1995).

Highly concentrated mucus had a blue tint and after centrifugation of worms, the mucus would separate into two layers, an upper blue layer and a lower translucent layer (Fig. 2B). It was not possible to completely separate the two layers from each other while in the liquid state, but our attempts showed that samples from both layers produced similar amounts of light. As the samples glowed, the mucus became less viscous and fully translucent, which happened within minutes after KCl stimulation. In *O. enopla*, luminous reagents that were partially purified had different colors, including yellowish and pink (Shimomura *et al.* 1963) but not blue. In an attempt to identify whether this color could be due to trace elements, the copper and iron concentrations of viscous mucus were analyzed using Induced Coupled Plasma Spectrometry (ICP-OES) following nitric acid treatment of mucus samples (Hanaoka *et al.* 2001). Mucus concentrations of $0.2\text{--}1.2 \times 10^{-1}$ ppm Cu and $0.2\text{--}6.4 \times 10^{-1}$ ppm Fe were greater than that of

seawater that contained non-stimulated worms, which had values below detection levels (10^{-5} ppm). Although the elements were more concentrated in mucus, there is no evidence that they contribute to the blue color or play a role in the bioluminescence reaction.

Monochromatic green emission spectrum. The bioluminescence emission spectrum of *O. phosphorea* was for the first time measured directly from live individuals. The emission spectrum after KCl stimulation appeared as a unimodal peak with maximum emission at 494–504 nm and full width at half maximum (FWHM) of about 50–60 nm (Fig. 3), with no apparent difference in emission spectra between male and female individuals (data not shown). Bioluminescence emission spectra of mucus stimulated with peroxidase or APS often appeared narrower and peaked between 494–498 and 494–504 nm, respectively (Fig. 3). The fluorescence emission spectrum of freshly KCl-stimulated mucus was unimodal and broad, peaking at 495–515 nm with a FWHM of about 70–80 nm (Fig. 3). Fluorescence after stimulation with peroxidase or APS had a slight short-wavelength shift in peak emission to 488–503 nm (Fig. 3). These results indicate that the light emitting compound preserved similar spectral characteristics for bioluminescence, chemiluminescence, and fluorescence, yet the fluorescence was emitted mainly from the compound after it had produced light and/or been oxidized. These results are comparable to those for the partially purified luminescence system of *O. enopla* that has peak emission at 507 nm (Shimomura *et al.* 1963).

Ontogenetic shift from internal to secreted bioluminescence. The smallest individuals collected were 0.5 mm in size, with only several segments visible, but clearly recognized by their cirri. At this early juvenile stage of development, there was minimal secreted bioluminescence

although the range of light intensity could be similar to that of larger 2.5 mm sized individuals known to secrete bioluminescence but which were likely to be immature based on their size even though they already featured the external characteristics of adult worms. Intensity and kinetics of light production in smaller individuals was variable in response to KCl stimulation (Fig. 4A). Integrated bioluminescence from specimens was similar across the size range investigated, as shown by model of the allometric variation that was represented by the relationship: $B = 1.3 \times 10^8 * e^{-35.2 * e^{-6.2 * S}}$ with $R^2 = 0.002$ and $P > 0.05$, which showed a 30-50 times factor of range of variation between light production from smallest and largest individuals, as indicated by the model (Fig. 4B). This suggests that the capability to produce light is innate to post-metamorphic worms and not acquired during subsequent growth, development, and sexual maturation (Tsuji and Hill 1983; Fischer and Fischer 1995). For worms ≤ 1 mm body length, the light intensity from ASW after the KCl-stimulated worms had been removed was negligible, with levels about 100 to 1000 times less than when the measurement was made with the worm present. These results indicate that the bioluminescence in these small worms was mainly internal. In contrast, even though larger (> 1 mm) worms had sometimes similar levels of integrated bioluminescence than the smaller individuals, removing the worm from the sample decreased the level of light only by a factor of 3 - 5, indicating that removal of the worm did not greatly affect the amount of bioluminescence being produced. Thus in the larger individuals the bioluminescence was mostly secreted from the body and ended up in the surrounding medium. Accordingly, the allometric variation of bioluminescence intensity in water that had contained a stimulated worm followed an exponential relationship: $B = 6.3 \times 10^7 * e^{-4740 * e^{-4.7 * S}}$, with $R^2 = 0.51$ and $P \leq 0.05$ (Fig. 4B).

The bioluminescence of *O. phosphorea* does not function solely in intraspecific behaviors related to swarming. Internal bioluminescence associated with startle defensive functions has

been reported during foraging behavior of adults and following mechanical stimulation (Fischer and Fischer 1995). The results of this study also supported the dual role of bioluminescence in interspecific/defensive and intraspecific/mate attraction behaviors. Small individuals (<1 mm), which were not reproductively mature and thus would not be involved in mating swarms, produced intense yet strictly internal bioluminescence. Accordingly, collector handling of the bryozoan *Zoobotryon verticillatum* during nighttime field sampling collection triggered a myriad of intense sparkles of light due to the mechanical stimulation of associated *O. phosphorea* found, using light microscopy, crawling on the bryozoan's surface; these small individuals only, and not the bryozoan, produced light as filmed with a low light video system (pers. obs.). Thus our data using total chemical depolarization with KCl to trigger light production clearly showed that in the 0.5 - 2.5mm size range, the larger the individual the more the bioluminescence is released as a luminous mucus, thus providing the worm with an additional form of light production to just internal bioluminescence. Finally, individuals kept in the aquarium for up to six months past the swarming period still showed a similar capacity of bioluminescence (data not shown), thus suggesting a bioluminescence function other than reproductive.

Using fluorescence microscopy, sources of bioluminescence were spread over most of the worm body surface (Fig. 5). Fluorescence was observed from inside epidermal cells (probably gland cells, according to Harvey 1952) that were homogeneously distributed from one segment to the other, and from around the head. Eyes, cirri, antenna, and palp did not show any fluorescence. Fluorescence was also particularly bright towards the posterior section of individuals where it was confined within cells forming a mass deep in the body. Further anatomical and microscope analysis is needed to identify the tissue origins of the luminescence/fluorescence.

Light production was not reconstituted by mixing hot and cold extracts. In bioluminescence systems that follow a luciferin-luciferase model, hot extracts denature the luciferase and preserve activity of the luciferin, while cold extracts exhaust the luciferin by oxidation and preserve activity of the luciferase. Recombining the hot and cold extracts then reconstitutes a significant amount of light production. In this study, there was no clear indication for significant reconstitution of bioluminescence activity by addition of hot extracts to cold extracts, and vice versa, in a luminometer. Mixing hot and cold extracts increased light emission by 3 to 10 times in 1 min compare to background prior to mixing. Mixing cold + cold, hot + hot, cold + ASW, and hot + ASW sometimes resulted in an increase in light emission, but only 1.2 to 1.5 times greater than background (Fig. 6). The intensity of reconstituted light from recombining hot and cold extracts always remained <3 % of the level of emission prior to the hot/cold treatments, much less than expected if the bioluminescence involved a luciferin-luciferase system (Harvey 1952). Addition of peroxidase 1 min later was predicted to trigger intense light production from combinations containing hot extract, indicating that this particular extract probably contained a reduced substrate (or chromophore) capable of producing light through an oxidation process. However, additional light production after peroxidase treatment was sometimes not observed with hot extracts, suggesting that the substrate was prematurely oxidized or subject to denaturation due to high temperature (Fig. 6). The mucus could also contain inhibitors to the luciferase activity and/or oxidized luciferin, in which case the hot-cold extract recombination tests might result in increased emission after dilution or dialysis of the cold extract to separate the oxidized luciferin or other inhibitors from the luciferase (Shimomura *et al.* 1963). These possibilities were not tested in this study. However, Harvey's (1952) qualitative observation that

“a good luminescence” was obtained by mixing hot and cold extracts without dialysis does not support the inhibitor hypothesis. Amidst these contradictory observations, the results of the present study suggest that *O. phosphorea* bioluminescence is not a luciferin-luciferase system.

Bioluminescence still occurred in oxygen-depleted conditions. Worms exposed to air bubbling occasionally produced bioluminescence, perhaps from mechanical stimulation when entrained in the bubbles. These worms, however, produced high levels of bioluminescence following KCl stimulation, though with high inter-worm variability. Following argon or nitrogen bubbling, worms sometimes produced more spontaneous light than the still control or air bubbled worms. Although the worms exposed to argon or nitrogen bubbling sometimes did not show external signs of life after treatment, KCl stimulation always resulted in a rapid increase in bioluminescence intensity, with light production stabilizing within an order of magnitude for all treatments (Fig. 7A). Therefore the results did not reveal a striking difference among treatments, suggesting that light production is not strictly dependent on the presence of molecular oxygen in the medium.

Luminous mucus exposed to bubbling still produced spontaneous bioluminescence, yet with levels different between treatments and experiments. Bioluminescence intensity from argon and nitrogen bubbled mucus was 1.2 to 10 times lower than that of still control and 10 to 90 times greater than that of air bubbled samples (Fig. 7B). Mucus still produced additional light when stimulated with APS, increasing to levels that were about 60 times the spontaneous bioluminescence in still control samples, 3 times greater in air bubbled samples, and 1.5 to 15 times greater in argon and nitrogen bubbled samples, respectively (Fig. 7B). From these results it is clear that mucus still produced bioluminescence under anoxic conditions, suggesting that the

reaction of light production does not directly requires oxygen. This conclusion was also supported by the observation that 10 mM sodium thiosulfate, a chemical with strong oxygen scavenger capacity, only inhibits light production by 30% while such a high concentration is predicted to fully inhibit bioluminescence if light production is strictly oxygen dependent {Wang, 2002 #3959}.

One explanation is that the bioluminescent system is efficient at very low oxygen concentrations. A more reasonable conclusion is that the emission of bioluminescence in oxygen-depleted conditions suggests that the bioluminescence in *O. phosphorea* involves a photoprotein rather than a luciferin/luciferase system. This conclusion disagrees with previous results with *O. enopla* showing that oxygen is necessary for light production (Shimomura et al. 1963). While it is possible that the two species (*O. phosphorea* and *O. enopla*) could have distinct reactions of bioluminescence, the discrepancy about the light production process may originate from the different nature of the samples used in the experiments, being fresh luminous mucus in this study and fractions of the luminescence system partially purified from hundreds of worms for the Shimomura *et al.* (1963) study.

Bioluminescence intensity decreased and decay rate increased with warmer temperature.

Bioluminescence of mucus was rapidly affected by temperature. For example, after 15 min of treatment, spontaneous bioluminescence intensity showed little difference between -20, 4, and 20 °C, while it was reduced to 0.1% at 40°C, and to 0.0001% at 60 and 80°C (Fig. 8). Mucus bioluminescence intensity increased upon treatment with APS as well as with subsequent exposure to peroxidase, always with greater extent for the APS stimulation. APS stimulated light production was greater for colder temperatures, being up to 90 times the level of spontaneous

bioluminescence at -20°C , and 8 times at 40°C , yet was similar at 60 and 80°C ; bioluminescence intensity increased 1.2 to 5 times after peroxidase treatment (Fig. 8).

The intensity of spontaneous bioluminescence from mucus decreased over time, reaching about 80% of the pre-treatment level after 30 min at -20°C , 45% when at 4°C , and 10% at 20°C ; the intensity reached $< 1\%$ in 20 min at 40°C , and within 5 min at 60 and 80°C (Fig 9A). The temperature effect on decrease in intensity of APS and peroxidase stimulated bioluminescence was similar to that of spontaneous bioluminescence (data not shown). The change in intensity with temperature always followed a simple exponential decay (Table 1). The decay rate increased gradually with temperature, showing the least changes between -20 and 20°C going from about 0.05 to 0.5 s^{-1} , and 60 and 80°C going from about 1.5 to 1.6 s^{-1} ; the greatest changes in decay rate were observed between 20, 40, and 60°C going from about 0.5 to 1.5 s^{-1} (Fig. 9B). These results suggest that the light production in *O. phosphorea* involves a reagent (enzyme, substrate or co-factor) that degrades rapidly, or stops to be involved in light production process, at temperatures greater than 20°C , this considering the level of bioluminescence intensity as a proxy for functional reagents involved in chemical reaction leading to light production.

The various mucus treatments (mucus, mucus + APS, mucus + APS + peroxidase) showed similar temperature effects on decay rate although the values for mucus samples were always lower than for the other treatments except at -20°C (Fig. 9B). In other words, bioluminescence intensity following APS and peroxidase treatment decreased at a faster rate than for untreated mucus at each corresponding temperature above -20°C . This probably resulted from the fact that APS and peroxidase treatments then stimulated relatively less bioluminescence at temperatures warmer based on the initial untreated spontaneous bioluminescence of the mucus. The difference between treated and untreated samples was greatest at 60 and 80°C .

Furthermore, the initial bioluminescence intensity (the α coefficient) was greater in APS and peroxidase treated mucus than for mucus alone, and was lowest at temperatures of 60 and 80°C (Table 1). These results suggest that a reagent or co-factor of the light production process degraded at temperatures of 60 and 80°C, thus becoming not or less available for optimal stimulation with APS and peroxidase at these higher temperatures.

Addition of APS resulted in a 10-15 fold increase in bioluminescence intensity for temperatures $\leq 40^\circ\text{C}$ (Fig. 8; Table 1). Assuming that APS acts only through protein-protein interaction (Fancy and Kodadek 1999; Denison and Kodadek 2004), these results suggest that the luminescent source in *O. phosphorea* is associated with a protein or apo-protein that is degraded by elevated temperature yet is well preserved at temperatures as low as -20°C . Addition of peroxidase to APS treated mucus approximately doubled the bioluminescence intensity (Fig. 8; Table 1). This suggests that the protein involved in light production process has a function similar to peroxidase activity.

Overall, the temperature results indicated a direct relationship between increasing temperature and decreasing light production, consistent with the hypothesis that the bioluminescence follows a process involving a photoprotein rather than a luciferin-luciferase system (Shimomura 1985; Shimomura 1986). For example, the photoprotein aequorin has been shown to maintain bioluminescence activity down to -75°C , yet is rapidly inactivated above 30°C {Shimomura, 1985 #2056}, similar to our results for *O. phosphorea*.

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Table 1: Parameters of the model for exponential decay of bioluminescence, $y = \alpha * e^{(-\beta * x)}$, where α is the intercept and β the decay rate, for various temperature treatments of mucus, mucus + ammonium persulfate (APS), and mucus + peroxidase. R^2 is the coefficient of determination, in bold for $P \leq 0.05$.

Stimulation	T (°C)	Model parameters		
		α	β	R^2
None (spontaneous)	-20	3.42×10^5	0.053	0.83
	4	3.87×10^5	0.152	0.99
	20	5.37×10^5	0.325	0.90
	40	8.81×10^5	0.893	0.97
	60	0.17×10^5	1.261	0.56
	80	0.22×10^5	1.293	0.60
APS	-20	4.68×10^6	0.042	0.59
	4	6.35×10^6	0.207	0.99
	20	11.01×10^6	0.499	0.94
	40	8.70×10^6	1.027	0.95
	60	0.09×10^6	1.568	0.53
	80	0.24×10^6	1.684	0.65
Peroxidase	-20	9.69×10^6	0.033	0.32
	4	13.61×10^6	0.276	0.95
	20	23.61×10^6	0.604	0.95
	40	15.56×10^6	1.043	0.96
	60	0.13×10^6	1.468	0.50
	80	0.95×10^6	1.592	0.72

Figure caption
(Deheyn and Latz)

Figure 1: Typical time course of bioluminescence of larger (female) individual (black line) and smaller (male) individuals (dark and light grey lines) of *Odontosyllis phosphorea*. The first 60 s of measurement were made with no stimulation to assess spontaneous bioluminescence. Light production was stimulated at 60 s by KCl, and at 180 s by peroxidase. Dashed line is the background level of the instrument.

Figure 2: Secreted mucus from 20 individuals of *Odontosyllis phosphorea*. (A) The mucus was highly viscous and developed threads when collected with pipette. (B) After centrifugation, the mucus separated into two layers, the upper layer blue and the lower translucent. The brown pellet contained the worms.

Figure 3: Relative intensity emission spectra of bioluminescence and fluorescence of *Odontosyllis phosphorea*. The intact worm was treated with KCl, while the mucus was treated with peroxidase and ammonium persulfate (APS). Spectra were smoothed as described in the methods and are offset for clarity. Dotted line marks 500 nm for reference. (A) Bioluminescence emission spectra, which peaks at 494-504 nm. (B) Fluorescence emission spectra with peaks between 488-515 nm, based on 380 nm excitation.

Figure 4: A. Typical time course of bioluminescence for 0.5 mm (light grey), 1.3 mm (dark grey) and 2.5 mm-long individuals of *Odontosyllis phosphorea*. B. Bioluminescence as a function of body size for juvenile individuals < 2.5 mm. Filled squares represent samples containing worm and seawater while open circles the corresponding sample from which the worm was removed, thus leaving mucus as the only source of bioluminescence. Allometric variations were fitted with an exponential model represented for the seawater only.

Figure 5: Juvenile individuals of *Odontosyllis phosphorea* viewed using (A) bright field and (B, C, D) epifluorescence microscopy. (C) Fluorescence originated from epidermal cells spread over the body segments. (D) Fluorescence from the head was not associated with eyes, palp, and antenna. Scale bar is 0.5 mm for A-B, 0.05 mm for C-D.

Figure 6: Experiment using hot/cold extracts of the mucus of *Odontosyllis phosphorea*. Spontaneous bioluminescence of an extract was measured for 10 s at which the complementary extract was added. Peroxidase was added to the mix 60 s later. Control experiments consisted in adding the same extract as the one already being measured, or ASW.

Figure 7: Effect of gas bubbling on the bioluminescence of *Odontosyllis phosphorea*. Treatments included the still control (light grey thick line), air bubbling (dark grey thick line), and argon or nitrogen bubbling (black lines). Chemical stimulation occurred after 60 s measurement of spontaneous light emission. (A) Individual worm treated with KCl. (B) Mucus treated with ammonium persulfate (APS). The dashed line is the background level for the luminometer.

Figure 8: Effect of temperature on bioluminescence from mucus of *Odontosyllis phosphorea* after 15 min treatment. Spontaneous bioluminescence was recorded before stimulation with ammonium persulfate (APS) at 10 s followed by stimulation with peroxidase at 20 s. Data are expressed in relative values compared to the first time point for the 20°C treatment.

Figure 9: Effect of temperature on bioluminescence of *Odontosyllis phosphorea*. (A) Spontaneous light emission of mucus; filled square for -20°C, open circle for 4 °C, inverted triangle for 20°C, filled circle for 40°C, open square for 60°C, and open triangle for 80°C. (B) Exponential decay rate (β) of bioluminescence from untreated mucus (square), mucus + ammonium persulfate (APS, triangle) and mucus + APS + peroxidase (circle).

Fig. 1

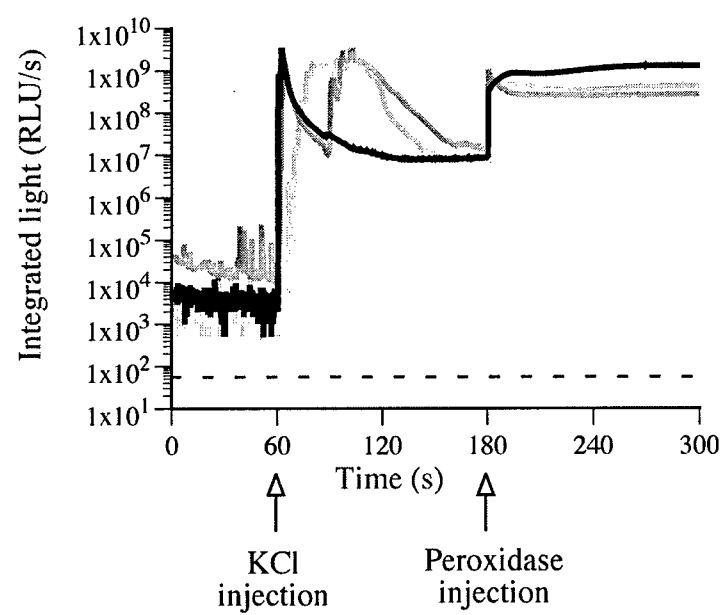


Fig 2

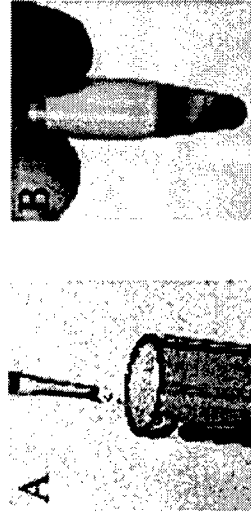


Fig. 3

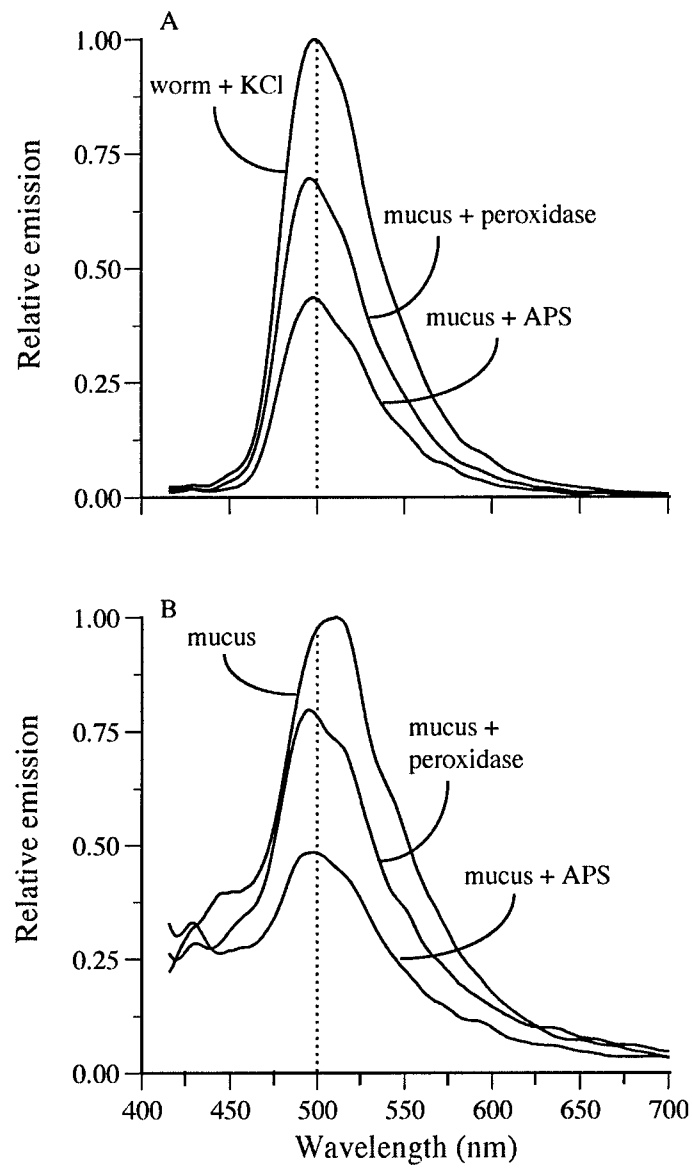


Fig. 4

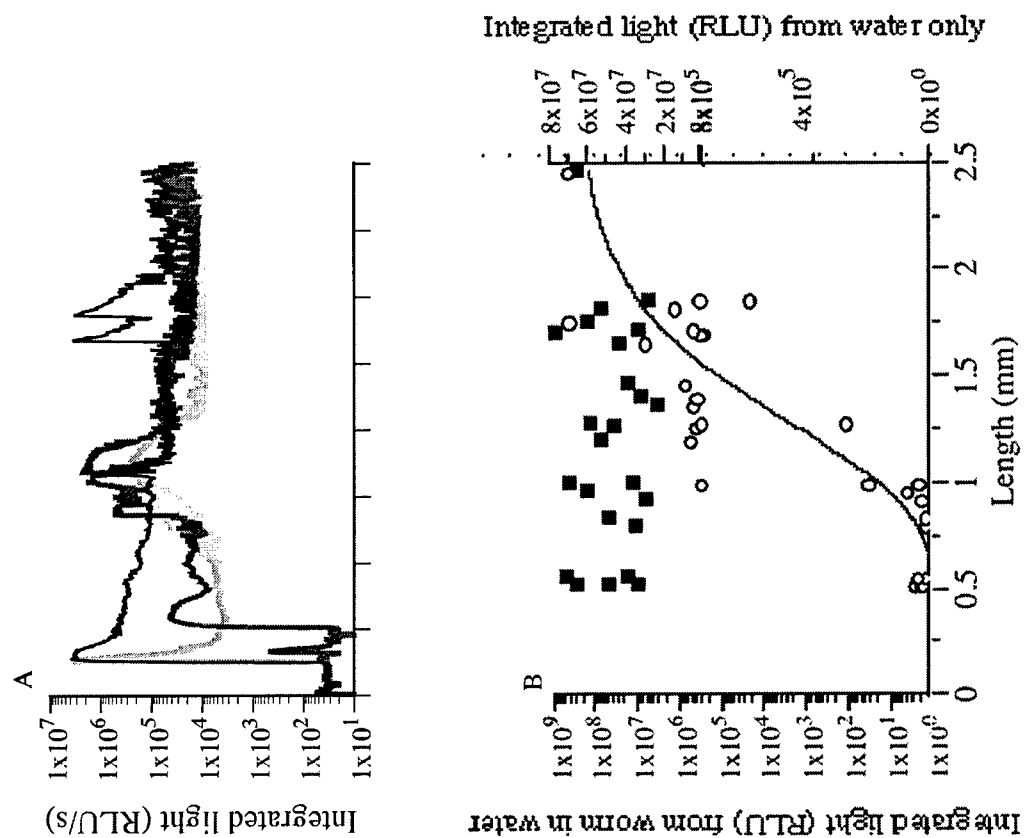


Fig. 5

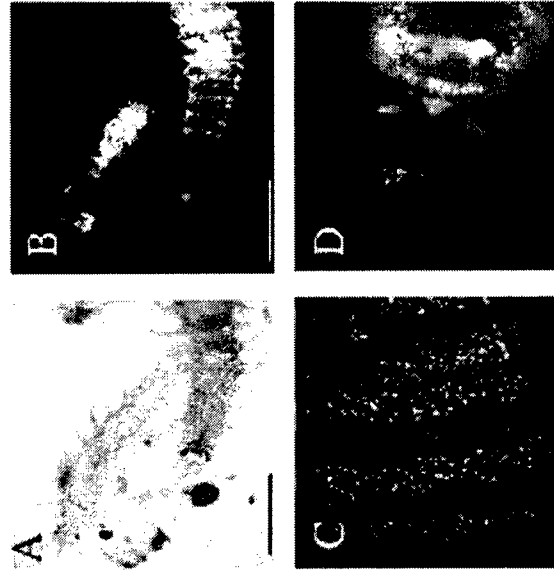


Fig. 6

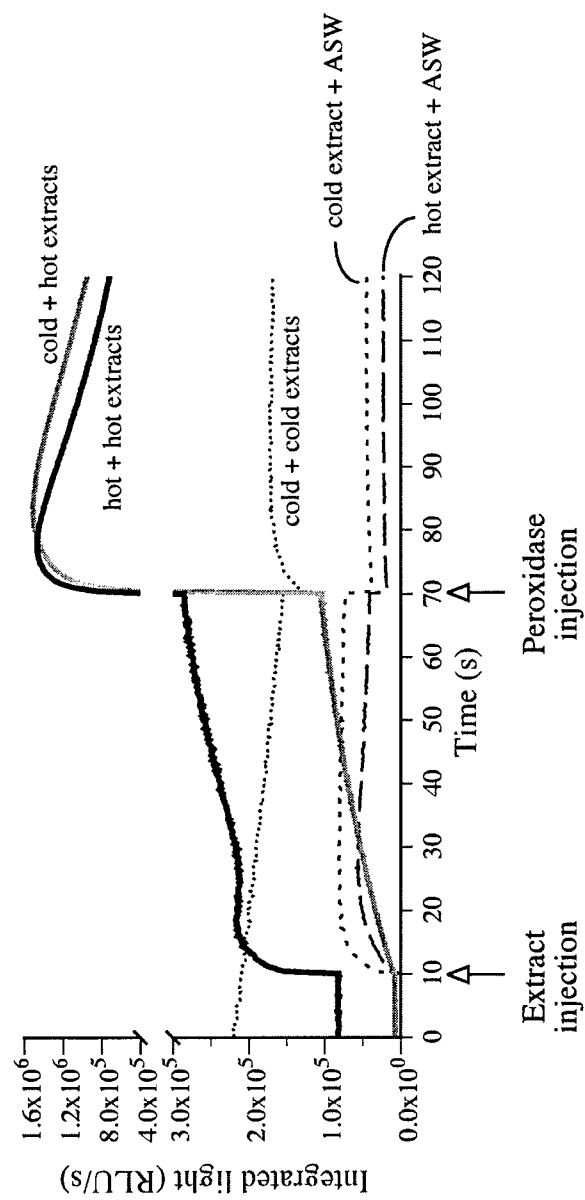


Fig. 7

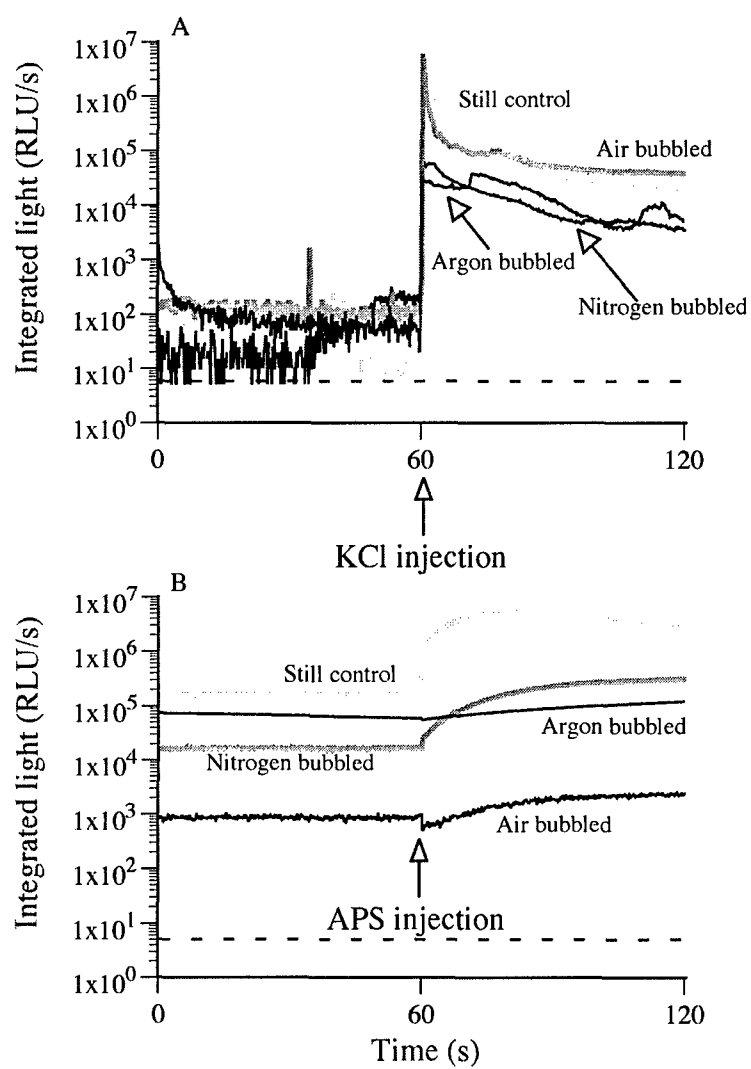


Fig. 8

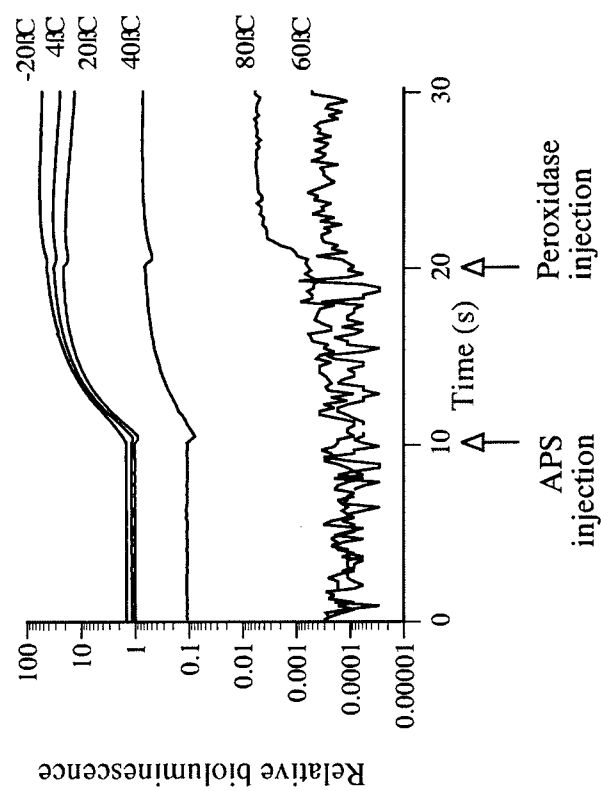


Fig. 9

